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The therapeutic effects of a pentacyclic triterpene derivative, bardoxolone methyl, in preventing high-fat diet-induced obesity and associated neural, hepatic, cardiovascular, and renal complications

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The Therapeutic Effects of a Pentacyclic Triterpene Derivative, Bardoxolone Methyl, in Preventing High-Fat Diet-Induced Obesity and Associated Neural, Hepatic, Cardiovascular, and Renal Complications

A thesis submitted in fulfilment of the requirements for the award of the degree

DOCTOR OF PHILOSOPHY

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By

Danielle Jocelyn Ana Camer, BCA, BMedSc Hons (Class I)

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Certification

I, Danielle Jocelyn Ana Camer, declare that this thesis, submitted in fulfilment of the requirements for the award of Doctor of Philosophy, in the School of Medicine, University of Wollongong, is entirely my work unless otherwise referenced or acknowledged. This manuscript has not been submitted for qualification at any other academic institution

Danielle Jocelyn Ana Camer

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Statements

In accordance with the University of Wollongong thesis committee 'Guidelines for Higher Degree Research Candidates on the Preparation and submission of Higher Degree Research Theses' (May 2014) this PhD is presented in 'Journal Article Style'. It is comprised of a series of four original studies under revision or published in peer-reviewed journals, of which I am the first author. I hereby declare that I am the primary designer of these studies, and have carried out experimental procedures, data analysis, and manuscript preparation.

Danielle Camer

I consent to the presentation of this PhD in 'Journal Article Style" and I acknowledge the above statement pertaining to student contribution to be correct.

Prof Xu-Feng Huang, Supervisor

Dr Yinghua Yu, Supervisor

Dr Alexander Szabo

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Publications

The following publications and presentations have arisen directly from work contained within this thesis.

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- **Camer D**, Yu Y, Szabo A, Fernandez F, Dinh HL C, Huang XF: Bardoxolone methyl prevents high-fat diet-induced alterations in prefrontal cortex signalling molecules involved in recognition memory. *Progress in Neuro-Psychopharmacology & Biological Psychiatry* 2015, 59: 68-75.
- **Camer D**, Huang XF: Is B-type Natriuretic Peptide a Risk Factor for Heart Failure in Patients Treated With Bardoxolone Methyl?. *Journal of Cardiac Failure* 2014, 21: 258-259.
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- **Camer D**, Huang XF: The Endothelin Pathway: A Protective or Detrimental Target of Bardoxolone Methyl on Cardiac Function in Patients with Advanced Chronic Kidney Disease? *Am J Nephrol* 2014, 40(3):288-290.

- **Camer D**, Yu Y, Szabo A, Huang XF: The molecular mechanisms underpinning the therapeutic properties of oleanolic acid, its isomer and derivatives for type 2 diabetes and associated complications. *Mol Nutr Food Res* 2014, 58(8):1750-1759.

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- **Camer D**, Bell CJ, Yu Y, Szabo A, Fernandez F, Dinh HL C, Huang XF**:** Bardoxolone Methyl: A Potential Therapeutic for the prevention of anti-psychotic drug-induced obesity?. *22nd International "Stress and Behavior" Neuroscience and Biopsychiatry Conference* 2015. St Petersburg, Russia.
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- **Camer D**, Yu Y, Szabo A, Dinh C, Fernandez F, Huang XF: Cognition can be improved with bardoxolone methyl treatment in mice fed a high-fat diet. *Australian Neuroscience Society 34th Annual Meeting* 2014. Adelaide, Australia.
- **Camer D**, Yu Y, Szabo A, Dinh C, Huang XF. The triterpene derivative bardoxolone methyl prevents the development of obesity and type 2 diabetes in mice. *International Diabetes Federation World Diabetes Congress* 2013. Melbourne, Australia.
- **Camer D**, Yu Y, Huang, XF: The therapeutic effects of an oleanolic acid derivative in diet induced obesity. *Australian Neuroscience Society 33rd Annual Meeting* 2013. Melbourne, Australia.

Additional Publications:

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- Dinh HL C, Szabo A, Yu Y, **Camer D,** Wang H, Huang XF: Bardoxolone methyl prevents mesenteric fat deposition and inflammation in high-fat diet mice. *The Scientific World Journal* 2015, In Press.
- Feng Y, Yu Y, Wang S, Ren J, **Camer D**, Hua Y, Zhang Q, Huang J, Xue D, Zhang X, Huang XF, Liu Y: Chlorogenic acid protects D-galactose-induced liver and kidney injury via antioxidation and anti-inflammation effects in mice. *Pharmaceutical Biology* 2015, In Press.
- Dinh HL C, Szabo A, Yu Y**, Camer D**, Zhang Q, Wang H, Huang XF: Bardoxolone methyl prevents fat deposition and inflammation in brown adipose tissue and enhances sympathetic activity in mice fed a high-fat diet. *Nutrients* 2015, 7(6):4705-23.
- Dinh HL C, Szabo A, **Camer D**, Yu Y, Wang H, Huang XF: Bardoxolone methyl prevents fat deposition and inflammation in the visceral fat of mice fed a high-fat diet. *Chemico-Biological Interactions* 2015, 229:1-8.
- Cusick A, **Camer D**, Stamenkovic A, Zaccagnini M: Supplementary instruction for research trainees using a peer assisted study session approach. *Journal of Peer Learning* 2015, In Press
- Cheng L, Yu Y, Szabo A, Wu Y, Wang H, **Camer D**, Huang XF: Increasing central palmitic acid induces leptin resistance and impairs hepatic glucose and lipid metabolism in mice. *Journal of Nutritional Biochemistry* 2015, 26(5): 541-8.

Oral Conference Presentation:

- O'Sullivan S, **Camer D**, Passfield J, Jackson A, Faricy C. Feedback for change and growth: PASS and the participant survey process. *8th National PASS forum 2012.* Melbourne, Australia (Invited Talk/Workshop).

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List of Abbreviations

ACC	Acetyl-CoA carboxylase
ACOX	Peroxisomal acyl-coenzyme A oxidase 1
AGE	Advanced glycation end product
AgRP	Agouti-related peptide
Akt	Protein kinase B
ALP	Alkaline phosphatase
AMPK	AMP-activated protein kinase
ANOVA	Analysis of variance
AR	Aldose reductase
ARE	Anti-oxidant response element
AST	Aspartate aminotransferase
AUC	Area under the curve
BDNF	Brain-derived neurotrophic factor
BM	Bardoxolone methyl
BMI	Body mass index
CCl_4	Carbon tetrachloride
CDDO	2-cyano- 3,12-dioxooleana-1,9(11)-dien-28-oic acid
CKD	Chronic kidney disease
CML	N^{ε}-(carboxymethyl) lysine
CSF	Cerebrospinal fluid
EDTA	Ethylenediaminetetraacetic acid
ET-1	Endothelin 1
ET_A	Endothelin receptor type a
ET_B	Endothelin receptor type b
FAS	Fatty acid synthase
FOXO1	Forkhead box protein O1
GCLC	Glutamate cysteine ligase catalytic subunit
GLUT4	Glucose transporter 4
G6P	Glucose 6 phosphate
G6Pase	Glucose-6-phosphatase
GK	Glucokinse
GSHpx	Glutathione peroxidase
GTT	Glucose tolerance test
$HbAl_c$	Glycated haemoglobin
H&E	Haematoxylin and Eosin
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
HF	High-fat
Hmox1	Heme oxygenase 1
HSD	Honestly significant difference
i.c.v	Intracerebroventricular
IκB	Nuclear factor kappa b inhibitor alpha
IKKβ	Inhibitor of nuclear factor kappa-B kinase subunit beta
IKKε	Inhibitor of nuclear factor kappa-B kinase subunit epsilon
IL-1β	Interleukin 1 beta
IL-6	Interleukin 6

iNOS	Inducible nitric oxide synthase
i.p	Intraperitoneal
IR	Insulin receptor
IRS	Insulin receptor substrate
IST	Insulin sensitivity test
JAK2	Janus kinase 2
JNK	c-Jun N-terminal kinase
Keap1	Kelch-like ECH-associated protein 1
LC	Lab chow
LPS	Lipopolysaccharide
LTD	Long term depression
LTM	Long term memory
LTP	Long term potentiation
NAFLD	Non-alcoholic fatty liver disease
NFκB	Nuclear factor kappa-B
NMDA	*N*-methyl-D-aspartate
NPY	Neuropeptide Y
Nqo1	NADPH dehydrogenase quinone 1
Nrf2	Nuclear factor-like 2
OA	Oleanolic Acid
PFC	Prefrontal cortex
PI3K	Phosphoinositide 3 kinase
POMC	Pro-opiomelanocortin
PTP1B	Protein tyrosine phosphatase 1B
ROS	Reactive oxygen species
RT-PCR	Quantitative Real Time PCR
SCD1	Stearoyl-CoA desaturase 1
SDH	Sorbitol dehydrogenase
SOCS3	Suppressor of cytokine signalling 3
SOD	Superoxide dismutase
STAT3	Signal transducer and activator of transcription 3
TNFα	Tumour necrosis factor alpha
TNFR	TNF receptor
TrkB	Tropomyosin related kinase B
UA	Ursolic acid
WAT	White adipose tissue

Abstract

The prevalence of obesity is a growing problem since it significantly increases the risk of developing type 2 diabetes and associated complications of the brain, liver, heart, and kidneys. Therefore, there is an urgency to find novel therapies which can prevent obesity and the development of associated complications. This PhD project investigated whether selected pentacyclic triterpenes (oleanolic acid (OA), its isomer, ursolic acid (UA), and derivative, bardoxolone methyl (BM)) administered at 10 mg/kg daily in drinking water could prevent obesity in mice fed a chronic HF diet for 21 weeks. These compounds were chosen based on recent studies demonstrating that they have a number of anti-obese and anti-diabetes properties. In preliminary studies, BM prevented HF diet-induced body weight gain, while UA and OA had no effect. Following this, the molecular mechanisms underlying the ability of BM to prevent HF diet-induced obesity and associated complications were then examined.

BM administration for 21 weeks prevented HF diet-induced increases in body weight, energy intake, plasma leptin, and peripheral fat (Chapter 2). Furthermore, in the mediobasal and paraventricular nuclei regions of the hypothalamus, BM treatment prevented HF diet-induced impairments of downstream leptin JAK2-Akt-FOXO1 signalling and increases in the inflammatory molecules, pJNK, TNFα and IL-6 . These findings identify a potential novel neuropharmacological application for BM to prevent HF diet-induced obesity, hypothalamic inflammation and leptin resistance.

BM administration also prevented HF diet-induced impairments in recognition memory (Chapter 3). Furthermore, in the hippocampus and prefrontal cortex (PFC), BM treatment prevented HF diet-induced decreases in downstream BDNF signalling molecules and increases in the inflammatory molecule, PTP1B. In summary, the findings from this chapter suggest that BM

prevents HF diet-induced impairments in recognition memory by improving downstream BDNF signal transduction, and reducing inflammation in the PFC and hippocampus.

BM treatment prevented HF diet-induced insulin resistance and hepatic steatosis in mice fed a HF diet (Chapter 4). Furthermore, in the livers of mice, BM prevented HF diet-induced impairments to hepatic IR-IRS-FOXO1 insulin signalling, *ACOX*-induced lipid metabolism, macrophage infiltration, and inflammation. These findings suggest that BM prevents HF diet-induced insulin resistance and the development of hepatic steatosis through modulation of molecules involved in insulin signalling, lipid metabolism, and inflammation in the liver.

BM administration prevented HF diet-induced structural changes in the heart and kidneys (Chapter 5). Furthermore, in these tissues, BM administration prevented HF diet-induced increases in fat accumulation, macrophage infiltration and *TNFα* gene expression. These findings suggest that BM prevents HF diet-induced developments of cardiac and renal pathophysiologies in mice fed a chronic HF diet by preventing inflammation.

Collectively, this thesis is novel in demonstrating that BM treatment prevents HF diet-induced obesity and associated leptin resistance, insulin resistance, cognitive deficits, and liver, kidney, and heart pathophysiologies in mice fed a HF diet for 21 weeks. These results suggest that these therapeutic effects were through anti-inflammatory mechanisms. Overall, these findings highlight BM as a potential novel therapeutic for preventing HF diet-induced obesity and a variety of associated complications.

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Chapter One

1.1 INTRODUCTION

Obesity is a growing health problem characterised by excessive fat accumulation and is defined as having a body mass index (BMI) exceeding or equal to 30 (WHO 2015). From 2011-12, over 63% of Australian adults were considered overweight or obese, which was a 5% increase from 1995 (ABS 2013). The prevalence of obesity globally is reaching epidemic proportions and has more than doubled since 1980 (WHO 2015). Worldwide, 1.9 billion people were considered overweight and 600 million people obese in 2014 (WHO 2015). Obesity also places economic burdens on society, with its estimated costs for 2008 in Australia reaching around $58 billion (AccessEconomics 2008). An elevated BMI as a result of excessive fat accumulation is a major risk factor for the development of a number of potentially life-threatening diseases such as type 2 diabetes and cardiovascular disease. This highlights the urgency of the need to find novel therapeutics that can prevent the development of high-fat (HF) diet-induced obesity and associated complications. Recent research has uncovered the molecular mechanisms responsible for the therapeutic properties of the pentacyclic triterpenes, oleanolic acid (OA), ursolic acid (UA), and their derivatives such as bardoxolone methyl (BM). In particular, recent reports have highlighted the benefits of these compounds in the prevention and treatment of obesity, type 2 diabetes, and associated life-threatening complications, such as non-alcoholic fatty liver disease, and nephropathy. Findings from *in vitro* and *in vivo* studies have demonstrated that these compounds improve insulin signalling and reduce hyperglycaemia, reduce oxidative stress by upregulating anti-oxidants, and reduce inflammation by inhibiting proinflammatory signalling. Therefore, these compounds were chosen for examination in the present PhD in order

to investigate their therapeutic effects in a HF diet-induced obese mouse model and to establish the most potent treatment for further investigation. In preliminary testing, prevention of body weight gain was only found in BM treated mice, and therefore, only this treatment group was investigated in detail in subsequent experimentation. Overall, this series of studies aimed to understand the molecular mechanisms underlying the therapeutic benefits of BM in preventing chronic HF diet-induced obesity, type 2 diabetes, and associated complications of the brain, liver, kidneys and heart. A better understanding of these mechanisms and therapeutic targets may assist in the use of BM as a future novel pharmaceutical for preventing and treating obesity and associated complications.

1.2 REVIEW OF LITERATURE

[Most of this literature review is based on: 1) our review: Camer et al. (2014). The molecular mechanisms underpinning the therapeutic properties of oleanolic acid, its isomer, and derivatives for type 2 diabetes and associated complications. *Mol Nutr Food Res* 2014, 58(8):1750-1759; and 2) our invited perspective: Camer et al. (2014). The Endothelin Pathway: A Protective or Detrimental Target of Bardoxolone Methyl on Cardiac Function in Patients with Advanced Chronic Kidney Disease? *Am J Nephrol* 2014, 40(3):288-290. (See Appendix 1.1 and 1.2)].

1.2.1 Obesity, Type 2 Diabetes, and Associated Complications

Obesity is a metabolic disorder that is characterised by a deregulation of energy balance, which is correlated to an increase in consumption of palatable HF food and reduced energy expenditure. The prevalence of obesity is a growing problem since it greatly increases the risk of developing type 2 diabetes (Kahn, Hull et al. 2006). Type 2 diabetes has reached epidemic proportions worldwide. Recent predictions indicate that the prevalence of diabetes globally will increase from 285 million in 2010 to 439 million in 2030 (Shaw, Sicree et al. 2010). Along with hyperglycaemia

and reduced insulin sensitivity, other characteristics featured in type 2 diabetes include proinflammation and oxidative stress, which contributes to tissue damage in organs such as the brain, liver, kidney, and heart. Obesity and associated type 2 diabetes can lead to the development and progression of a number of potentially life-threatening complications, including cognitive deficits, hepatic steatosis, chronic kidney disease, and heart disease (Forouhi and Wareham 2010). Therefore, identifying new medicinal agents, especially derived from natural products, offers exciting possibilities for future development of successful therapeutics to prevent diet-induced obesity, type 2 diabetes, and associated complications.

1.2.2 Obesity-Induced Hypothalamic Leptin Resistance

Leptin is an adipokine produced by adipocytes in adipose tissue. In a normal functioning state, downstream hypothalamic leptin-JAK2-Akt-FOXO1 signalling allows the suppression of hunger signals causing satiety (Friedman and Halaas 1998, Elmquist, Elias et al. 1999, Bates and Myers 2003). In the hypothalamus, leptin binds to long form leptin receptors and functions to regulate food intake and energy expenditure via neuronal interactions known as central leptin signalling (Friedman and Halaas 1998, Elmquist, Elias et al. 1999, Bates and Myers 2003). Central leptin administration via intracerebroventricular (i.c.v) injection results in an increase of janus kinase 2 (JAK2) and protein kinase b (Akt) phosphorylation (Roman, Reis et al. 2010). However, an i.c.v central leptin injection results in an increase in the phosphorylation of Akt in lean mice but not obese mice, which suggests that central leptin resistance may be caused by a deregulation in phosphorylated (p) Akt and leptin signalling (Metlakunta, Sahu et al. 2008). Furthermore, pAkt can subsequently inactivate forkhead box protein O1 (FOXO1), a transcription factor in the hypothalamus (Kim, Pak et al. 2006). Inactivation of FOXO1 leads to downregulation of neuropeptide Y (NPY) and agouti-related peptide (AgRP) but upregulation of pro-opiomelanocortin (POMC) expression, therefore leading to negative energy balance (Morton,

Gelling et al. 2005, Kim, Pak et al. 2006, Plum, Lin et al. 2009). In obesity, this pathway is largely impaired due to accentuated activation of the negative regulators, protein tyrosine phosphatase 1B (PTP1B) and suppressor of cytokine signalling 3 (SOCS3), which inhibit JAK2 and Akt activation respectively (Bence, Delibegovic et al. 2006, Zhang, Zhang et al. 2008). Thus, if there is a dysfunction in this pathway, this regulation of food intake and energy expenditure is disabled.

There is compelling evidence that hypothalamic inflammation is a key characteristic of obesity in rodents and humans (Cai and Liu 2011). Recent research has demonstrated that a HF diet results in low grade hypothalamic inflammation in rodents (Thaler, Yi et al. 2012). Furthermore, within a week of starting a HF diet, rodents have increased mRNA expression of the proinflammatory cytokines, tumour necrosis factor alpha (TNFα) and interleukin 6 (IL-6) in the hypothalamus (Thaler, Yi et al. 2012). Hypothalamic inflammation leads to the development of central leptin resistance through activation of PTP1B, an inhibitor of leptin signalling (Zhang, Zhang et al. 2008, Milanski, Arruda et al. 2012). Obesity from a HF diet has been demonstrated in both rodents and humans to cause central leptin resistance which limits the clinical effectiveness of exogenous leptin administration (Caro, Kolaczynski et al. 1996, Nam, Kratzsch et al. 2001). Central leptin resistance has been suggested to occur since it has been found that although leptin levels in the cerebrospinal fluid (CSF) is 30% higher in obese individuals than individuals with a lean body mass, the CSF NPY levels are not reduced (Caro, Kolaczynski et al. 1996, Nam, Kratzsch et al. 2001). Therefore, novel therapeutics that target hypothalamic inflammation and downstream leptin signalling may have the potential to prevent the development of obesity and leptin resistance.

1.2.3 Obesity-Induced Cognitive Decline in the Forebrain

Obesity is a major risk factor for the development of cognitive decline in neurodegenerative diseases such as vascular dementia (Hassing, Johansson et al. 2002). A number of studies

provide direct evidence demonstrating a link between HF diet-induced obesity and impairments in learning and memory performance, including a decline in recognition memory (Greenwood and Winocur 1990, Greenwood and Winocur 1996, Heyward, Walton et al. 2012). Furthermore, preclinical animal studies have demonstrated that a HF diet reduces synaptic plasticity in the prefrontal cortex (PFC) (Val-Laillet, Layec et al. 2011) and hippocampus (Molteni, Barnard et al. 2002, Wu, Molteni et al. 2003), which leads to learning and memory impairments (Laroche, Davis et al. 2000). A HF diet can further induce cognitive decline by promoting neuroinflammation in the forebrain (Miller and Spencer 2014). Despite this, therapeutic interventions targeting HF diet-induced cognitive impairment are lacking.

Obesity-induced cognitive impairment is attributed to a reduction of synaptic plasticity (Molteni, Barnard et al. 2002). Recent evidence has indicated that HF diet-induced impairment in neuronal plasticity may be caused by the reduction of brain derived neurotrophic factor (BDNF) protein expression in the PFC and hippocampus, which are key brain areas in learning and memory (Kanoski, Meisel et al. 2007). BDNF signalling is a critical pathway for promoting long term potentiation (LTP), a form of synaptic plasticity associated with long term memory (LTM) formation, and neurogenesis in the forebrain (Noble, Billington et al. 2011). Tropomyosin related kinase B (pTrkB) receptor phosphorylation and activation by BDNF leads to a downstream intracellular cascade resulting in activation and phosphorylation of Akt signalling (Cunha, Brambilla et al. 2010). Akt signalling regulates the translation and transport of synaptic proteins in order to promote synaptic plasticity in learning and memory (Yoshii and Constantine-Paton 2007).

Along with the activation of TrkB, BDNF also triggers the opening of Na^+ gated ion channels, resulting in an influx of Ca^{2+} and the enhancement of glutamate activation of *N*-methyl-D-aspartate (NMDA) receptors (Rose, Blum et al. 2004). NMDA receptors also play a crucial role in synaptic plasticity with their activation by glutamate, leading to the induction of LTP (Bliss and

Collingridge 1993, Cooke and Bliss 2006). A previous study has reported that a HF diet desensitises NMDA receptors in the hippocampus in mice causing impairment in NMDA-induced long term depression (LTD), suggesting that its alteration may also account for cognitive defects (Valladolid-Acebes, Merino et al. 2012). Another important signalling protein that is linked to BDNF is phosphorylated AMP-activated protein kinase (pAMPK). Studies have demonstrated that pAMPK activation increases BDNF expression in the brain (Gomez-Pinilla, Vaynman et al. 2008, Yoon, Oh et al. 2008, Zhao, Shen et al. 2008), suggesting that its activation plays a crucial role in promoting synaptic plasticity. Furthermore, it has been reported that a HF diet reduces phosphorylation of AMPK in the hippocampus in rats (Wu, Ying et al. 2006).

It is widely accepted that consumption of a HF diet and obesity leads to obesity-induced chronic inflammation in a number of tissues, including the brain (Weisberg, McCann et al. 2003, Xu, Barnes et al. 2003). Several rodent studies have demonstrated that chronic inflammation in the brain induced by a HF diet is also associated with a decline in cognitive performance (Morrison, Pistell et al. 2010, Pistell, Morrison et al. 2010, Singh, Gupta et al. 2012). In the forebrain, synaptic plasticity is disrupted by increased expression of the inflammatory mediators, PTP1B (Fuentes, Zimmer et al. 2012) and phosphorylated c-Jun N-terminal kinase (pJNK) (Jiang, Yin et al. 2013). Therefore, novel therapeutics that target inflammation and promote downstream BDNF signalling in the hippocampus and prefrontal cortex has the potential to prevent the development of obesity-induced memory decline.

1.2.4 Obesity-Induced Insulin Resistance and Fat Accumulation in the Liver

Obesity is a major risk factor for the development of insulin resistance, type 2 diabetes, and hepatic steatosis (Kahn, Hull et al. 2006, Forouhi and Wareham 2010). It is widely accepted that HF diet-induced obesity causes increased fat accumulation, macrophage infiltration, and chronic inflammation in peripheral tissues (Weisberg, McCann et al. 2003, Xu, Barnes et al. 2003).

Increased fat accumulation and inflammation promotes insulin resistance and tissue injury in peripheral tissues involved in glucose and fat metabolism, such as the liver (Weisberg, McCann et al. 2003, Xu, Barnes et al. 2003). A number of studies provide direct evidence demonstrating a link between obesity-associated inflammation and insulin resistance, and hepatic steatosis (Ginsberg 2006, Qureshi and Abrams 2007, Emanuela, Grazia et al. 2012). However, there is a need to develop novel therapeutic approaches targeting hepatic inflammation and to prevent obesity-induced insulin resistance and hepatic steatosis.

The activation of inflammatory pathways can promote the expression of the negative regulators of insulin signalling, PTP1B and SOCS3 (Hong, Nguyen et al. 2001, Zabolotny, Kim et al. 2008). PTP1B levels are increased in the liver of HF diet-induced obese mice, which contributes to the development of insulin resistance by reducing insulin signalling through inhibition of insulin receptor (IR) and insulin receptor substrate 1 (IRS-1) activation (Goldstein, Bittner-Kowalczyk et al. 2000, Lam, Covey et al. 2006). SOCS3 is another important molecule which impairs insulin signal transduction in the liver through its inhibition of the binding of IR to IRS-1 (Ueki, Kondo et al. 2004). Furthermore, activation of hepatic insulin signalling results in the inactivation of FOXO1, which is a transcription factor that inhibits the transcription of genes such as *glucose-6-phosphatase (G6Pase)* for endogenous glucose production via gluconeogenesis (Nakae, Kitamura et al. 2001, German, Kim et al. 2009). When insulin signalling is impaired, through inhibition by PTP1B or SOCS3, and activation of FOXO1, this leads to the promotion of glucose production and a reduction in glucose reuptake, leading to glucose intolerance and insulin resistance in obesity (Nakae, Kitamura et al. 2001, German, Kim et al. 2009). BDNF also plays an import role in insulin action, as it has been found to modulate hepatic glucose metabolism via its actions on glucokinse (GK) in obese insulin resistant rats (Kuroda, Yamasaki et al. 2003). In the

liver, GK enhances glycolysis, resulting in reduced blood glucose levels (Hariharan, Farrelly et al. 1997).

A HF diet is known to cause fat accumulation in the liver, which can progressively worsen to hepatic steatosis (Marchesini, Brizi et al. 2001). Hepatic lipid homeostasis is regulated by a number of genes that promote lipogenesis, including acetyl-CoA carboxylase (*ACC)*, *fatty acid synthase (FAS)* and *stearoyl-CoA desaturase 1 (SCD1)*, and β oxidation, such as *peroxisomal acyl-coenzyme A oxidase 1 (ACOX)* (Musso, Gambino et al. 2009). Hepatic fat accumulation leads to macrophage infiltration which promotes the production of proinflammatory cytokines, such as IL-6, TNFα and interleukin-1 beta (IL-1β) (McArdle, Finucane et al. 2013). Increased IL-6 has been found to enhance inflammatory signalling by increasing signal transducer and activator of transcription 3 (STAT3) levels, which promotes cytokine dependent signalling by increasing the expression of inflammatory genes such as IL-6 (Yang, Liao et al. 2007). In addition, inhibitor of nuclear factor kappa-B kinase subunit beta (IKKβ), and inhibitor of nuclear factor kappa-B kinase subunit epsilon (IKKε) are important proinflammatory signalling molecules upstream of the transcription factor, nuclear factor kappa-B (NFκB), which promote PTP1B and SOCS3 activation (Hong, Nguyen et al. 2001, Zabolotny, Kim et al. 2008, Napetschnig and Wu 2013). Therefore, novel therapeutics that target inflammation, and promote lipid metabolism and downstream hepatic insulin signalling in the liver may have the potential to prevent the development of obesity-induced insulin resistance and hepatic steatosis.

1.2.5 Obesity-Induced Development of Cardiorenal Diseases

Obesity caused by the consumption of a HF diet increases the risk of cardiorenal diseases. Cardiovascular disease is the leading cause of death worldwide, with the incidence expected to rise from 17.3 million per year in 2008 to over 23.6 million per year by 2030 (Mozaffarian, Benjamin et al. 2015). There is increasing evidence that obese individuals have an increased risk

of developing cardiovascular disease (Kenchaiah, Evans et al. 2002). In addition, there is direct evidence that obesity from a HF diet can cause kidney injury, which also increases the associated cardiovascular disease risk (Prasad 2014). Therefore, there is an urgent need to find suitable therapeutics that can prevent HF diet-induced obesity-associated complications of the heart and kidney, in order to reduce the incidence of global mortality from cardiorenal diseases.

The endothelin system has been suggested to play an important role in the development of cardiovascular pathophysiologies. In the heart, endothelin 1 (ET-1) acts through two receptors, endothelin receptor type a (ET_A) and endothelin receptor type b (ET_B). The key endothelin system molecules ET-1, ET_A, and ET_B play a role in vasoconstriction, with ET_B also having an additional role in vasodilation (Kedzierski and Yanagisawa 2001). In the cardiac muscle, ET-1 activates ET_A which results in the promotion of cardiac hypertrophy leading to subsequent heart failure (Nasser and El-Mas 2014). Previous studies have demonstrated that there is therapeutic potential in targeting the endothelin system with ET_A or combined ET_A/ET_B antagonists in patients with congestive heart failure (Krum, Viskoper et al. 1998, Nakov, Pfarr et al. 2002). However, it is important to note that in the kidneys the endothelin pathway plays several important roles including the regulating sodium and water homeostasis and renal blood flow (Kohan 2006). Therefore, over-suppression of the endothelin pathway by antagonistic drugs may lead to other complications in the kidneys such as fluid retention, which if not addressed can also lead to heart failure (Kohan 2006).

Obesity from HF diet is known to result in the development of fat accumulation in peripheral organs, such as the heart and kidneys (Montani, Carroll et al. 2004). Furthermore, peripheral fat accumulation is associated with macrophage infiltration into adipose tissue, which promotes the release of proinflammatory cytokines including TNFα (Wellen and Hotamisligil 2005). In a recent study, significantly higher levels of inflammatory markers, including TNFα, were found in the

cardiac tissue of Tibetan mini pigs as a result of being fed a HF diet for 24 weeks (Yongming, Zhaowei et al. 2015). Furthermore, rats fed a HF diet for 10 weeks demonstrated increased TNFα levels in the cortex of their kidneys (Elmarakby and Imig 2010). Therefore, novel pharmaceuticals that attenuate TNFα levels and appropriately target the endothelin pathway in the heart and kidneys is warranted in order to prevent obesity-associated cardiovascular disease and renal failure.

1.2.6 Oleanolic Acid (OA), Its Isomer, and Derivatives

Both oleanolic acid (OA) and its isomer, ursolic acid (UA) exist widely in nature and can be extracted from fruits, herbs and vegetables. OA can be found in olive leaves, olive pomace, mistletoe sprouts and clove flowers, whilst UA can be found in apple pomace. A mixture of these two triterpenes can also be found in rosemary leaves (Jager, Trojan et al. 2009). Both OA and UA are pentacyclic triterpenes, which is a group of widespread natural compounds containing six isoprene units, the basic molecular formula C30H48 and with five rings in their skeleton (Jager, Trojan et al. 2009, Patočka 2012) (Figure 1.1). Recently, OA and UA have received great attention because of their benefits including anti-hyperglycaemic, anti-hyperlipidemic, anti-inflammatory, and anti-oxidative properties, with potential application for the treatment of obesity, type 2 diabetes and associated complications (Huang, Yang et al. 2005, Jayaprakasam, Olson et al. 2006, Gao, Li et al. 2009, de Melo, Queiroz et al. 2010, Kela, Srinivasan et al. 2010, Pergola, Raskin et al. 2011, Rao, de Melo et al. 2011, Wang, Li et al. 2011). Although they differ only in the position of a side chain in their structure, a number of *in vitro* and animal studies have demonstrated that OA and UA exhibit different degrees of potency in particular functions including their direct binding to insulin signalling molecules, such as PTP1B (Liu 1995, Huang, Peng et al. 2005, Wang, Hsu et al. 2010, Ramirez-Espinosa, Rios et al. 2011).

Triterpenoids are structurally similar to steroids and may, like steroids, diffuse freely through cell membranes to interact with intracellular molecular targets. The semi-synthetic triterpenoid 2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oic acid (CDDO) has been developed along with chemically modified derivatives containing various functional groups on rings A and C (Honda, Rounds et al. 1998, Honda, Rounds et al. 2000). These novel compounds are far more potent than natural triterpenoids and can affect signalling pathways in mammalian cells that are associated with detoxification, inflammation, and apoptosis (Suh, Honda et al. 1998, Yore, Liby et al. 2006). These sites can be accentuated and manipulated through chemical modification of the natural compound into derivative form. Examples of highly potent synthetic OA derivatives are the CDDO derivatives, which are strong anti-oxidant compounds. In particular, the highly potent OA CDDO derivative, bardoxolone methyl (BM) highlights the promising potential of these compounds (Figure 1.1). BM has successfully completed phases I and II of human clinical trials (Pergola, Krauth et al. 2011, Pergola, Raskin et al. 2011) and is currently recruiting patients for future clinical studies for patients with type 2 diabetes and chronic kidney disease (NCT02316821), and patients with pulmonary arterial hypertension (NCT02036970).

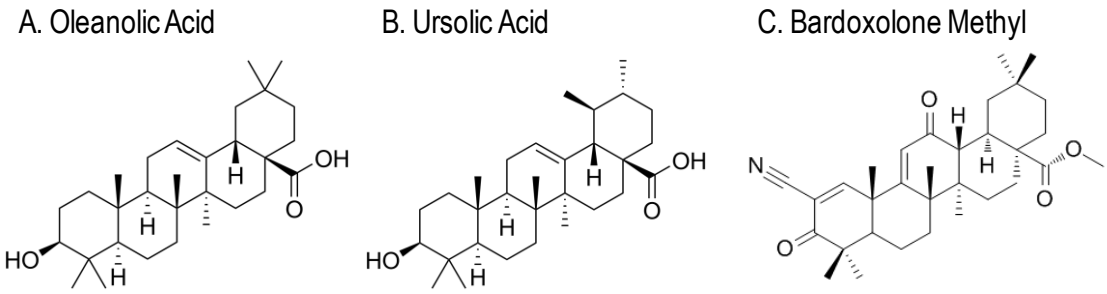

Figure 1.1. The molecular structures of selected pentacyclic triterpenes and a derivative. (A) Oleanolic Acid, (B) Ursolic Acid, and (C) Bardoxolone Methyl.

1.2.7 Therapeutic Effects of OA, Its Isomer and Derivatives in Body Weight and Blood Glucose Regulation

UA and OA have been found to reduce plasma triglyceride and total cholesterol levels, and reduce visceral adiposity and body weight in rodents fed a HF diet (de Melo, Queiroz et al. 2010, de Melo, Queiroz et al. 2010, Jang, Kim et al. 2010, Rao, de Melo et al. 2011). Several studies have demonstrated the ability of OA and UA in normalising blood glucose levels in rodents with diet-induced obesity or diabetes (Jayaprakasam, Olson et al. 2006, Gao, Li et al. 2009, Jang, Yee et al. 2009, de Melo, Queiroz et al. 2010, Ramirez-Espinosa, Rios et al. 2011, Rao, de Melo et al. 2011). In particular, in two preventative studies where mice were administered OA or UA at a dosage of 10 mg/kg in conjunction with being fed a HF diet for 15 weeks, blood glucose levels were significantly lower compared to non-triterpene HF diet-fed controls by 37% and 42% respectively (de Melo, Queiroz et al. 2010, Rao, de Melo et al. 2011).

Recent evidence suggests that OA, UA, and a number of their derivatives can improve insulin signalling by enhancing IR β subunit phosphorylation and Akt *in vitro* (Zhang, Hong et al. 2006, Jung, Ha et al. 2007, Lin, Zhang et al. 2008). Insulin regulates glucose homeostasis through binding of its receptor to initiate a signalling cascade; activation and phosphorylation of the IRS proteins, and mediation of the phosphatidylinositol 3-kinase-dependent/Akt (PI3K/Akt) pathway. The activation of the Akt pathway can 1) mediate the translocation of glucose transporter 4 (GLUT4) to the plasma membrane, thereby facilitating glucose uptake into adipose tissue, cardiac muscle and skeletal muscle (Haruta, Morris et al. 1995, Cong, Chen et al. 1997, Hajduch, Alessi et al. 1998, Charron, Katz et al. 1999, Wang, Somwar et al. 1999, Smith, Carvalho et al. 2000), and 2) inhibit glucose production via gluconeogenesis by glucose 6 phosphate (G6P) and FOXO1 in the liver.

OA and UA promote glucose uptake from the bloodstream into peripheral tissues through upregulation of GLUT4 [27-32]**.** In an *in vitro* study, UA promoted glucose uptake by enhancing the translocation of GLUT4 to the plasma membrane in 3T3-L1 adipocytes (Jung, Ha et al. 2007).

3T3-L1 adipocytes treated with an OA derivative, NPLC441, had increased GLUT4 mRNA and protein expression compared to untreated cells indicating increased glucose uptake into the cells (Lin, Zhang et al. 2008, Zhang, Zhang et al. 2008). A glucose uptake assay in L6 myotubes revealed that a 1 μM dose of an OA derivative increases basal glucose uptake by 40% (Lin, Zhang et al. 2008, Zhang, Zhang et al. 2008).

Another mechanism of OA and UA in lowering blood glucose is by the reduction of endogenous glucose production via inhibition of gluconeogenesis in the liver. Glucose production via gluconeogenesis can exacerbate hyperglycaemic states, and favours the development and progression of type 2 diabetes. Key molecules in the gluconeogenic pathway are G6Pase and FOXO1 (Yunoki, Sasaki et al. 2008, Jang, Kim et al. 2010). A 0.05% UA supplement decreased G6Pase activity and significantly elevated the hepatic glycogen content in the livers of streptozotocin and HF diet-induced diabetic mice (Yunoki, Sasaki et al. 2008, Jang, Kim et al. 2010). Adding 0.05% OA extracted from dietary wine pomace in HF diet significantly downregulated the mRNA expression of G6P (49%) and FOXO1 (52%) in liver of rats (Yunoki, Sasaki et al. 2008, Jang, Kim et al. 2010).

PTP1B has been proposed as a novel target whose inhibition would specifically address insulin resistance. PTP1B is a molecule that negatively regulates insulin signalling (Na, Oh et al. 2006, Lin, Zhang et al. 2008, Ramirez-Espinosa, Rios et al. 2011). Several *in vitro* studies have provided evidence that OA, UA and a number of their derivatives can directly inhibit PTP1B and improve insulin sensitivity (Na, Oh et al. 2006, Lin, Zhang et al. 2008, Ramirez-Espinosa, Rios et al. 2011). In particular, a PTP1B inhibition assay concluded that OA and UA adhered to the linear mixed type inhibition model in their interaction with PTP1B (Ramirez-Espinosa, Rios et al. 2011). Interestingly, the binding site of PTP1B targeted by OA and UA was uncovered to be a secondary region, known as site B rather than the typical catalytic binding site A (Ramirez-Espinosa, Rios et

al. 2011). This suggests that compounds that have high specificity for this region should be developed, such as through modifying OA and UA to derivative forms in order to achieve strong PTP1B inhibition and subsequent maximal improvement to insulin signal transduction. In addition, the OA and UA derivatives (C-28 addition) were more potent than their natural structures by 22 and 10 fold in inhibition of PTP1B activity respectively (Zhang, Hong et al. 2006, Zhang, Zhang et al. 2008). PTP1B can inhibit the PI3K/Akt signalling pathway to induce insulin resistance by inhibition of the translocation of GLUT4 to the plasma membrane. This causes disinhibition of FOXO1, thereby promoting reduction of glucose reuptake and gluconeogenesis (Sun, Wang et al. 2007). Therefore, the direct inhibition of PTP1B by OA, UA and their derivatives enables signal transduction of insulin and thus improves insulin sensitivity.

In addition to insulin sensitisation, inhibition of PTP1B also has the potential to promote weight loss, which is a benefit since obesity largely contributes to the type 2 diabetes pathology. Oral administration of OA, UA and their derivatives decreased body weight gain in HF diet-induced obese rodents (Jayaprakasam, Olson et al. 2006, Gao, Li et al. 2009, Jang, Yee et al. 2009, de Melo, Queiroz et al. 2010, Ramirez-Espinosa, Rios et al. 2011, Rao, de Melo et al. 2011). PTP1B-deficient mice were resistant to weight gain and remained insulin-sensitive when subjected to a HF diet, while the amount of food consumed was not different (Bence, Delibegovic et al. 2006). The increased insulin sensitivity of PTP1B knockout mice cannot explain the reduced weight gain on a HF diet (Bence, Delibegovic et al. 2006). Several *in vitro* studies demonstrated that PTP1B is a negative regulator of the leptin-JAK2-STAT3 signalling pathway. Therefore, competitive inhibitors of PTP1B, such as OA, UA and their derivatives might have the potential as a future novel therapeutic for obesity and type 2 diabetes. A summary of the effects of UA and OA on PTP1B inhibition of PI3K/Akt insulin signalling is presented in Figure 1.2.

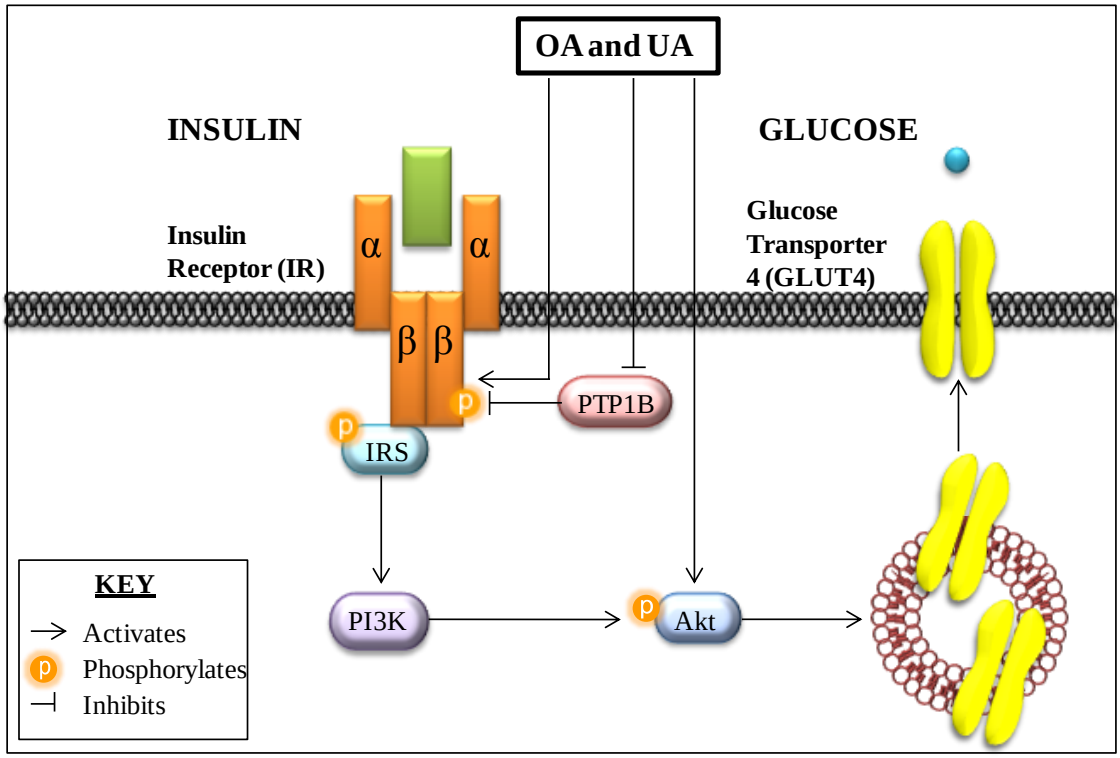

Figure 1.2 Ursolic acid (UA) and oleanolic acid (OA) effects on insulin signalling and glucose uptake in the target cell. Insulin binds to the insulin receptor (IR) at the α subunits resulting in a conformational change. Tyrosine residues on the β subunits phosphorylate resulting in downstream insulin signalling. PTP1B inhibits insulin signalling. Insulin receptor substrate (IRS) proteins are activated resulting in subsequent activation of phosphoinositide 3 kinase (PI3K) and protein kinase B (Akt). In peripheral tissues including cardiac muscle, skeletal muscle and adipose tissue, Akt activates the translocation of glucose transporter 4 (GLUT4), which is sequestered in vesicles before activation, into the plasma membrane. GLUT4 facilitates glucose uptake into the cell. Oleanolic acid (OA) and ursolic acid (UA) treatment inhibits PTP1B by directly binding to site B. This causes improved insulin signal transduction.

1.2.8 Therapeutic Effects of OA, Its Isomer, and Derivatives in Reducing Inflammation

Overnutrition leads to an accentuated proinflammatory state in several tissues including the liver and the hypothalamic region of the brain (Osborn and Olefsky 2012). Inflammation of these tissues contributes to hyperglycaemia, insulin resistance, and type 2 diabetes (Osborn and Olefsky 2012). On a molecular level, proinflammatory signalling is mediated by NF-κB activation. The proinflammatory NF-κB signalling pathway in the target cell is summarised in Figure 1.3. Briefly, NF-κB remains inactive when bound to and inhibited by nuclear factor kappa b inhibitor alpha (IκB) in the cytoplasmic region of the cell. NF-κB is activated by IKK, which stimulates its translocation into the nucleus. Once NF-κB is in the nucleus, it regulates the expression of a variety of molecules such as the cytokine, TNFα. The secretion of TNFα due to the activation of

NF-κB can also increase the production of reactive oxygen species (ROS), contributing to the development and progression of co-morbidities associated with obesity and type 2 diabetes, such as cardiovascular disease (Gao, Belmadani et al. 2007). This proinflammatory signalling pathway is a positive feedback loop since TNFα can bind to TNF receptor (TNFR) resulting in the phosphorylation and activation of IKK then subsequent NF-κB interaction. NF-κB activation can also promote the expression of the negative regulators of insulin signalling, PTP1B and SOCS3, thereby reducing insulin sensitivity and subsequent glucose regulation [61-64].

OA and UA have been found to reduce proinflammation by inhibiting proinflammatory signalling molecules and cytokines (Feingold, Soued et al. 1989, Grunfeld and Feingold 1991, Zhang, Zhang et al. 2008). A summary of OA and UA's effects on proinflammatory signalling in the target cell is summarised in Figure 1.3. OA reduced NF-κB signalling by inhibiting lipopolysaccharide (LPS) induced phosphorylation of IkB, and subsequently the expression of the cytokines TNFα and IL-1β (Suh, Jin et al. 2007, Yunoki, Sasaki et al. 2008). UA administration in mice fed a HF diet also inhibited signalling through the NF-κB pathway (Lu, Wu et al. 2011). The OA derivative, BM has been found to directly influence proinflammatory signalling in Human U-937 myeloid leukaemia cells by inhibiting IKK which causes blocking of the NF-κB pathway (Ahmad, Raina et al. 2006). This OA derivative has also been found to suppress LPS-induced inflammation in normal human PBMC cells by reducing the expression of the cytokines IL-6 and TNFα (Thimmulappa, Fuchs et al. 2007). However, a very high concentration of BM was required to suppress NF-κB in macrophages, suggesting that NF-κB signalling is not the only target by this compound and that its effects may occur through another pathway, possibly through nuclear factor like 2 (Nrf2) (Ahmad, Raina et al. 2006, Liby and Sporn 2012). Therefore, UA, OA and their derivatives such as BM appear to be promising compounds in targeting obesity-induced inflammation.

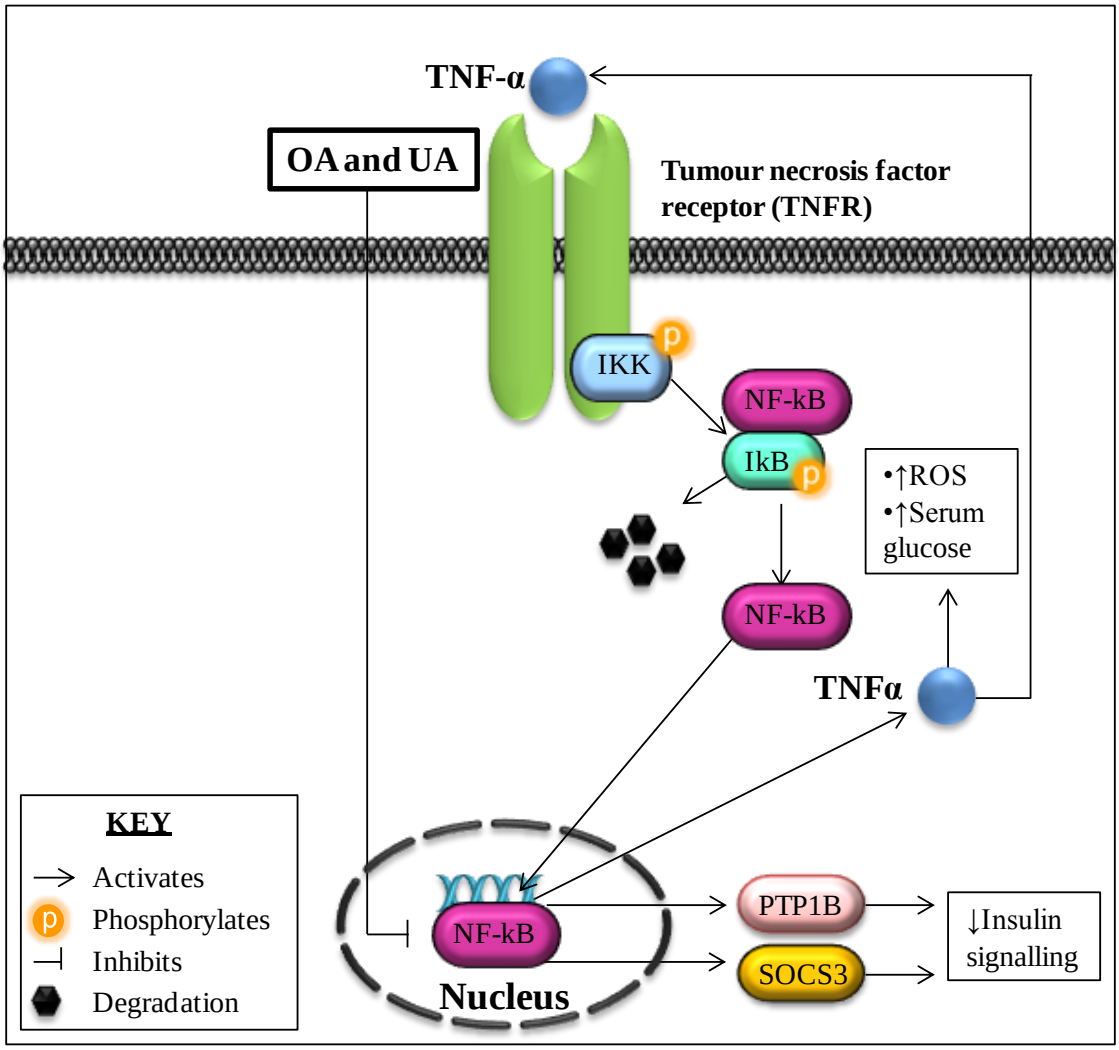

Figure 1.3. Ursolic acid (UA) and oleanolic acid (OA) effects on proinflammatory signalling in the target cell. Tumour necrosis factor alpha (TNFα) binds to the TNF receptor (TNFR). IκB kinase (IKK) is phosphorylated causing nuclear factor kappa b inhibitor alpha (IκB) phosphorylation. Disassociation of IκB from nuclear factor kappa B (NF-κB) and subsequent degradation. NF-κB is translocated into the nucleus where it functions to activate the transcription of a variety of molecules. NF-kB regulates the expression of TNFα, protein tyrosine phosphatase 1B (PTP1B) and suppressor of cytokine signalling 3 (SOCS3), which are negative regulators of insulin signalling. TNFα can then bind to the TNFR causing a detrimental feedback loop. UA has been found to inhibit NF-κB activation in mice whilst OA has been found to reduce NF-κB translocation into the nucleus by inhibiting IκB phosphorylation.

1.2.9 Therapeutic Effects of OA, Its Isomer, and Derivatives in Reducing Oxidative Stress and Tissue Damage

In type 2 diabetes, hyperglycaemia promotes an increase in free radicals and decrease in anti-oxidants causing increased lipid peroxidation. Free radicals such as ROS can be detrimental since they can diffuse into cells causing damage to the mitochondrial enzymes and DNA, which subsequently leads to cellular dysfunction (Gao, Li et al. 2009). ROS are generated by oxidative

stress such as the conversion of sorbitol to fructose in the polyl pathway (Jang, Kim et al. 2010). In particular, ROS have been found to play a role in kidney fibrosis (Dendooven, Ishola et al. 2011, Truong, Gaber et al. 2011). A study has shown that damaged tubular cells in kidneys exacerbate ROS leading to apoptosis following unilateral ureteral obstruction (Chung, Yoon et al. 2014).

One of the complications of type 2 diabetes is hepatocellular enzyme leakage, indicated by an increase in plasma enzyme activity of aspartate aminotransferase (AST) and alkaline phosphatase (ALP), which eventually results in severe liver damage (Wang, Li et al. 2011). A hepatoprotective effect of OA has been observed in mice with diabetes through a reduction in the activity of ALP and AST, suggesting a reduction in hepatoxicity (Wang, Li et al. 2011). In animal studies, OA and UA treatment decreased liver damage caused by oxidative stress-inducing chemicals, such as carbon tetrachloride (CCl_4) (Liu, Liu et al. 1994). OA and UA also increased the activities of the anti-oxidant enzymes superoxide dismutase (SOD) and glutathione peroxidase (GSHpx) (Ma, Zhao et al. 1986, Liu, Liu et al. 1994, Wang, Li et al. 2011). Increased levels of these anti-oxidant enzymes reduced the levels of free radicals and lipid peroxidation (Wang, Li et al. 2011). The anti-oxidative effects of these compounds appear to be beneficial for the treatment and prevention of associated complications of obesity and type 2 diabetes, including oxidative stress-induced liver damage.

Excess glucose in the blood promotes renal and hepatic tissue damage, and the polyl pathway is a major contributor to this damage. The function of this pathway is to metabolise unused glucose and it is activated during hyperglycaemic states. The key enzymes in this pathway are aldose reductase (AR) and sorbitol dehydrogenase (SDH), which facilitate the production of sorbitol and fructose. The elevated sorbitol and fructose levels that occur due to the polyl pathway results in an increase in advanced glycation end product (AGE) formation and glycative injury (Kawasaki,

Fujii et al. 1999, Takeuchi and Yamagishi 2004, Tokita, Hirayama et al. 2005). AGEs such as glycated haemoglobin ($HbAI_c$), N^{ε}-(carboxymethyl) lysine (CML), and glycated albumin are thought to be involved in the development of diabetic nephropathy, with CML and glycated albumin shown to contribute to its progression (Gugliucci and Bendayan 1996, Schleicher, Wagner et al. 1997, Ziyadeh, Mogyorosi et al. 1997, Dunlop 2000). OA and UA administration in mice with diabetes has been found to reduce the renal and liver activity of AR and SDH and mRNA expression of AR causing suppression of the polyl pathway via decreased sorbitol and fructose production and AGE formation (Jang, Kim et al. 2010, Wang, Hsu et al. 2010). OA treatment can also upregulate mRNA expression of glyoxalase I, an enzyme that metabolises the AGE precursor methylglyoxal (Beisswenger, Howell et al. 2003, Wang, Hsu et al. 2010). The suppression of these molecules integral to the polyl pathway and inhibition of AGEs, including precursors, by OA and UA ameliorates liver and kidney injury (Jang, Kim et al. 2010). This may hinder the progression of type 2 diabetes related complications of the liver and kidneys, including diabetic nephropathy, chronic kidney disease (CKD), and non-alcoholic fatty liver disease (NAFLD).

Nrf2 promotes the transcription of many anti-oxidant genes (Chen and Kunsch 2004, Li and Nel 2006, Wang, Ye et al. 2010), and its intracellular interactions are summarised in Figure 1.4. Nrf2 is usually bound to its inhibitor kelch-like ECH-associated protein 1 (Keap1) in the cytoplasm. An increase in oxidative or electrophillic stress-inducing agents such as ROS causes Keap1 to lose its ability to inhibit Nrf2 which results in the disassociation of Nrf2 from Keap1 (Itoh, Tong et al. 2004). Nrf2 can then be translocated into the nucleus where it binds to the anti-oxidant response element (ARE) to stimulate the transcription of anti-oxidant genes (Nguyen, Yang et al. 2004, Yu and Kensler 2005, Wei, Gong et al. 2011). The activation of Nrf2 and concurrent inactivation of Keap1 results in a reduction of oxidative stress and inflammation in a variety of tissues including

the kidneys, liver and retina. Nrf2 activation results in a reduction of blood urea nitrogen levels and the amelioration of glomerular and tubular injury in the kidneys (Wu, Wang et al. 2011). In the livers of knockout and knockdown Keap1 mice, Nrf2 activation causes reduced expression of hepatic inflammatory genes including IL-1β, IL-6 and TNFα (Liu, Wu et al. 2013). Following induced retinal ischemia reperfusion, Nrf2 knockout mice have been found to have increased inflammatory cells, increased inducible nitric oxide synthase (iNOS) and oxidative stress compared to wild type mice (Wei, Gong et al. 2011). In addition, Keap1 has been shown to promote a proinflammatory response through binding with the p65 subunit of NF-κB in HepG2 and HEK293 cells (Yu, Li et al. 2011). Furthermore, Nrf2 has been found to be activated as a result of NF-κB-induced inflammation and ROS production as a defensive response (Kim, Cha et al. 2010, Singh, Vrishni et al. 2010). This suggests that Nrf2 activation influences both inflammation and oxidative stress. OA, UA and derivatives have been found to have anti-inflammatory and anti-oxidative effects, which may be credited to Nrf2 activation. Briefly, the mechanisms behind this effect include inhibition of proinflammatory signalling and increasing the transcription of anti-oxidants; both of which are associated with Nrf2 activation.

OA and a number of synthetic derivatives of OA including BM, CDDO-TFEA, CDDO-Im and CDDO-Ea have been found to activate Nrf2 signalling [15,72, 74]. OA has been found to increase Nrf2 activation and heme oxygenase 1 (Hmox1) expression causing a reduction in fibrosis and apoptosis in mice with unilateral ureteral obstruction (Chung, Yoon et al. 2014). BM and CDDO-TFEA attenuate retinal damage, such as in diabetic retinopathy, via Nrf2 activation and subsequent transcription of several anti-oxidant genes including Hmox1, NADPH dehydrogenase quinone 1 (Nqo1) and glutamate cysteine ligase catalytic subunit (GCLC) (Pitha-Rowe, Liby et al. 2009, Wei, Gong et al. 2011). The treatment of retinal ischemia reperfusion-induced mice with BM increased retinal superoxide levels and reduced capillary degeneration by 60%. In addition to

decreasing retinal damage, CDDO-Im has been found to induce the phosphorylation of Akt in retinal epithelial cells. On inhibition of the PI3K/Akt pathway, CDDO-Im treatment had no effect in inducing Hmox1 transcription, reiterating the relationship between Akt activation and Hmox1 expression (Pitha-Rowe, Liby et al. 2009). This demonstrates a link between Nrf2 and Akt signalling pathways, and supports the previously described effect of these compounds on insulin signalling. This also suggests that the anti-oxidative and anti-inflammatory effects of Nrf2 activation may also be influenced by the activity of the PI3K/Akt pathway, perhaps through inhibition of PTP1B. Therefore, Nrf2 activation by OA derivatives appears to be a promising target for reducing oxidative stress in type 2 diabetes and associated complications, such as diabetic nephropathy and liver dysfunction.

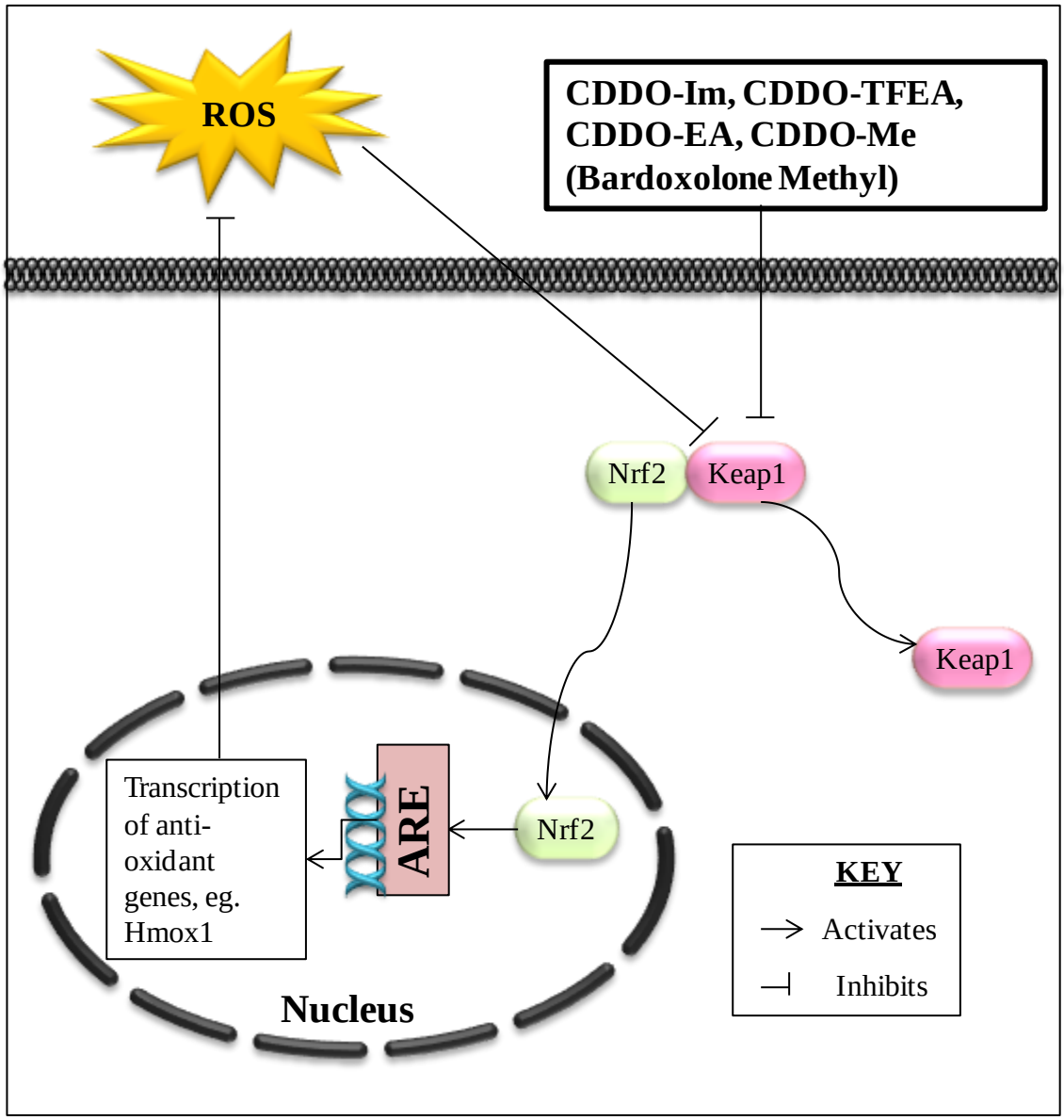

Figure 1.4. The effects of oleanolic acid (OA) CDDO derivatives on nuclear factor like 2 (Nrf2) activation. Oxidative stress such as reactive oxygen species (ROS) reduces the inhibitory activity of Kelch-like ECH-associated protein 1 (Keap1) on Nrf2. Nrf2 disassociates from Keap1 resulting in subsequent translocation of Nrf2 into the nucleus. Nrf2 binds to the anti-oxidant response element (ARE) to promote the transcription of a number of anti-oxidant genes such as Hmox1. Anti-oxidant enzymes transcribed by Nrf2 can inhibit ROS. The OA CDDO derivatives, CDDO-Im, CDDO-TFEA, CDDO-EA and CDDO-Me (Bardoxolone methyl) activate Nrf2 by reducing Keap1 inhibition of Nrf2.

1.2.10 Clinical Applications of OA and an OA Derivative, Bardoxolone Methyl (BM)

OA is currently used as a dietary supplement in traditional Chinese medicine for treating liver injuries, inflammatory diseases, various types of cancers, and diabetes (Liu 1995, Sultana and Ata 2008, Petronelli, Pannitteri et al. 2009, Wang, Li et al. 2011, Pollier and Goossens 2012). However, investigation of highly potent OA derivatives, including the OA CDDO derivative, BM, is

still currently undergoing several human clinical trials to test its potential future use in a clinical setting. BM has successfully completed phase I and II of human clinical trials for treating CKD in individuals with type 2 diabetes, and phase I clinical trials for the treatment of leukaemia and solid tumours (Liby, Yore et al. 2007), indicating its potential in treating multiple diseases. The phase IIb human clinical trial study, in 227 patients with type 2 diabetes and CKD, showed that BM improved kidney function with no sign of hepatic injury (Pergola, Raskin et al. 2011). The therapeutic effects of BM were through upregulation of Nrf2 and Hmox1 expression in various regions of the kidneys (Wu, Wang et al. 2011). Since BM has successfully completed phase II of human clinical trials with positive benefits in patients with CKD and type 2 diabetes, this compound has potential clinical applications in the treatment of kidney disease in type 2 diabetes. The ability of BM to activate Nrf2 may reduce oxidative stress and inflammation in other tissues such as the liver, thereby ameliorating tissue damage in individuals with obesity-induced type 2 diabetes and prevent the development of associated microvascular and macrovascular complications. Further scientific investigation of the effect of BM is needed in the future to determine if this drug has a similar effect in promoting Nrf2 activation in other tissues and organs and whether another molecular target, such as PTP1B is responsible for its therapeutic effects.

Despite the number of benefits of OA and the OA derivative BM, caution for specific populations should be taken when applied to patients with severe chronic kidney, hepatic and/or heart diseases. In a clinical setting, the dose of OA can be as high as 80mg three times per day in humans (Lu, Wan et al. 2013). However, caution must be taken since it has been reported that OA can cause hepatotoxicity in long term use or if the dose is too high (>500μmol/kg per day) in mice (Lu, Wan et al. 2013). Phase III of human clinical trials testing BM in patients with end stage chronic kidney disease (stage 4 and up) was terminated due to a higher incidence of cardiovascular events compared to the placebo group (de Zeeuw, Akizawa et al. 2013). The

mechanisms contributing to these adverse events in the clinical trial were speculated to be via the modulation of the endothelin pathway (Chin, Reisman et al. 2014). However, this pathway was not investigated in the heart tissue and in the kidney following chronic BM treatment, suggesting that further investigation into this drug was vital (Camer and Huang 2014). Therefore, future human clinical trials using BM should monitor blood pressure and heart function of participants, and overall caution should be taken in patients with a higher risk of cardiovascular events. Recruitment for a human clinical trial in patients with pulmonary arterial hypertension (NCT02036970) is currently being undertaken in order to determine the efficacy and safety of BM in this population, which is proposed to be completed by April 2016. This will aim to address safety issues in the phase III human clinical trials in advanced chronic kidney disease patients.

1.2.11 Therapeutic Effects of BM in Obesity and Associated Complications

OA is a natural compound that has shown a number of therapeutic benefits in the treatment and prevention of obesity and associated complications such as type 2 diabetes (Camer, Yu et al. 2014). In several *in vitro* studies, OA has been found to improve leptin signalling by activating the phosphorylation of Akt and reducing the leptin signalling inhibitor, PTP1B (Na, Oh et al. 2006, Jung, Ha et al. 2007, Lin, Zhang et al. 2008, Ramirez-Espinosa, Rios et al. 2011). However, derivatives of OA have been found to be significantly more potent and have a higher bioavailability than in their natural form (Zhang, Zhang et al. 2008). An example of a highly potent OA synthetic derivative is BM, which has attracted wide attention due to its anti-inflammatory effects and its potential application in a wide variety of diseases (Ahmad, Raina et al. 2006, Wang, Garvin et al. 2011, Liby and Sporn 2012, Reisman, Chertow et al. 2012, Camer and Huang 2014, Camer, Yu et al. 2014). In recent years, BM has been extensively studied in both preclinical rodent studies and human clinical trials, and shows promise for the treatment of renal diseases such as chronic kidney disease, and colitis-induced colon cancer due to its anti-

inflammatory effects (Liby, Yore et al. 2007, Pergola, Krauth et al. 2011, Pergola, Raskin et al. 2011, Hong, Kurzrock et al. 2012, NIH 2012, de Zeeuw, Akizawa et al. 2013, Camer, Yu et al. 2014, Choi, Kim et al. 2014).

Interestingly, a decrease in body weight and appetite were reported as side effects of BM in the phase II human clinical trials in a population with CKD and type 2 diabetes (Pergola, Raskin et al. 2011, NIH 2012). Furthermore, a recent study demonstrated that 2 week acute administration of BM decreased body weight in diet-induced obese mice (Saha, Reddy et al. 2010). In addition, previous studies have demonstrated that oral administration of a derivative of BM, 1-[2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oyl]imidazole (CDDO-Im), prevents HF diet-induced obesity and attenuates diabetes in mice (Shin, Wakabayashi et al. 2009, Uruno, Furusawa et al. 2013). However, the mechanisms behind these effects and whether it can prevent obesity-induced associated complications have not been investigated previously. Therefore, in Chapters 2 and 4 of this study, it was examined whether chronic treatment with BM could also prevent body weight gain, increased food intake and insulin resistance in mice fed a chronic HF diet. Furthermore, in Chapters 2 and 4 it was investigated whether BM could also prevent the development of hypothalamic leptin resistance and hepatic insulin signalling as potential mechanisms to explain its anorexigenic and blood glucose lowering effects respectively.

Although beneficial effects of BM have been demonstrated in animal models and human clinical trials in a variety of tissues (Pitha-Rowe, Liby et al. 2009, Pergola, Raskin et al. 2011), no study has investigated the effects of BM on the brain *in vivo*. It has been demonstrated that administration of OA has been found to reverse recognition memory impairments in mice (Park, Lee et al. 2014). Moreover, a recent study found that BM can promote dopaminergic neuroprotection via attenuation of the inflammatory mediator, TNFα, and ROS production *in vitro* (Tran, McCoy et al. 2008). Furthermore, a derivative of BM, CDDO-MA, improved spatial memory

and reduced hippocampal amyloid plaques in a mouse model of Alzheimer's disease (Dumont, Wille et al. 2009). Since these previous studies suggest that BM has therapeutic effects on the brain, in Chapter 3 of this thesis, it was investigated whether chronic BM treatment can prevent HF diet-induced decline in recognition memory and synaptic plasticity in the hippocampus and prefrontal cortex.

It is well established that BM directly influences the activity of proinflammatory signalling (Ahmad, Raina et al. 2006). Specifically, studies have demonstrated that BM can reduce inflammation by modulating TNFα levels in rodents fed a HF diet (Saha, Reddy et al. 2010, Dinh, Szabo et al. 2015). Therefore, in Chapters 4 and 5 of this thesis, it was investigated whether BM could also prevent injury of the liver, heart, and kidneys induced by HF diet-induced fat accumulation, macrophage infiltration and inflammation.

1.3 AIMS

1.3.1 General Aims

To examine the effects of BM treatment in mice fed a chronic HF diet for 21 weeks and to uncover the molecular mechanisms behind its therapeutic effects in preventing the development of obesity and associated type 2 diabetes, hepatic steatosis, cognitive deficits, and cardiorenal diseases.

1.3.2 Specific Aims

The specific aims of this research were:

1. To investigate the effects of BM in preventing deregulation of energy balance and hypothalamic leptin signalling in mice fed a HF diet.

2. To examine the effects of BM in preventing recognition memory decline and alterations in downstream BDNF signalling molecules in the hippocampus and PFC of mice fed a HF diet.

3. To examine the effects of BM in preventing the development of diet-induced insulin resistance, hepatic steatosis, and impaired downstream hepatic insulin signalling in mice fed a HF diet.

4. To determine if BM has protective or detrimental effects in obesity-induced cardiac and renal pathophysiologies in mice fed a HF diet.

1.3.3 Hypotheses

1. BM will prevent body weight gain, peripheral fat accumulation and leptin resistance by modulating hypothalamic leptin signalling in mice fed a chronic HF diet.

2. BM administration will prevent the development of obesity-induced recognition memory decline by modulating downstream BDNF signalling in the hippocampus and PFC of mice fed a chronic HF diet.

3. BM administration will prevent the development of obesity-induced insulin resistance and hepatic steatosis by modulating hepatic insulin signalling, inflammation and lipid metabolism in mice fed a HF diet.

4. BM administration will prevent the development of obesity-induced development of cardiac and renal pathophysiologies in mice fed a HF diet by decreasing inflammation.

1.3.4 Significance

Obesity and the associated development of type 2 diabetes have reached epidemic proportions worldwide. This increasing incidence is concerning as obesity and type 2 diabetes can lead to the development and progression of a number of potentially life-threatening complications affecting a variety of tissues including the brain, liver, kidneys and heart. Complications of obesity and type 2 diabetes include impaired energy balance regulation, memory deficits, hepatic steatosis, and cardiorenal diseases. Therefore there is an urgent need to find novel therapeutics that have the ability to prevent the development of obesity, type 2 diabetes and associated complications.

In human clinical trials, BM, a highly potent OA derivative, has demonstrated a therapeutic potential through its anti-inflammatory and anti-oxidative properties. In addition, reductions in body weight and appetite have been reported as side effects in patients taking the drug. Therefore, exploring the effects of BM on body weight regulation in mice fed a chronic HF diet will provide a novel understanding of the mechanisms underlying its therapeutic effects. Furthermore, the results from the present study will provide an insight into whether BM can also prevent obesity-induced type 2 diabetes and associated complications of the brain, liver, heart, and kidneys. These results may lead to identifying BM as a potential therapeutic for preventing and treating obesity, type 2 diabetes, and associated complications. Importantly, these findings may lead to the development of additional human clinical trials and the future use of BM as a novel pharmaceutical in the clinic.

1.4 GENERAL METHODS

1.4.1 Ethics Statement

This study was approved by the Animal Ethics Committee, University of Wollongong (Application Approval #: AE12/15), and all experimental procedures complied with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (2004), which is in accordance with the International Guiding Principles for Biomedical Research Involving Animals. All efforts were made to minimise animal stress and suffering.

1.4.2 Animals, Diet and Drug Treatment

Seventy male adult (12 week old) C57BL/6J mice were obtained from the Animal Resource Centre (Perth, Western Australia) and were maintained in the animal facility at the University of Wollongong, Wollongong, NSW, Australia. Mice were housed individually in environmentally controlled conditions (temperature 22 °C, 12hr light/dark cycle). Following 1 week of acclimatisation, mice were randomly divided into 5 groups (n=14 per group). For the next 21 weeks, one group of mice were fed a lab chow (LC) diet (5% of energy as fat; Vella Stock Feeds, Doonside, New South Wales, Australia), while the other four groups were fed a HF diet (40% of energy as fat; SF11-095, Specialty Feeds, Glen Forrest, Western Australia). The diet compositions of the LC and HF diets are presented in Table 1.1. Mice fed a HF diet were randomly assigned to UA, OA, or BM treatment groups, or the control group. For the treatment groups, a daily oral dosage of 10 mg/kg of UA, OA or BM administered in drinking water was chosen according to previous studies (de Melo, Queiroz et al. 2010, Rao, de Melo et al. 2011, Wu, Liu et al. 2014). Body weight and food intake were measured weekly for the duration of the experiment.

Table 1.1 Composition of the high-fat and lab chow diets

	High-fat diet	Lab chow diet
Total energy (kcal/100g)		
Fat	40	5
Carbohydrate	45	75
Protein	15	20

Typical Ingredients;
High-fat diet: Casein (Acid), Sucrose, Lard, Sunflower Oil, Cellulose, Wheat Starch, Dextrinised Starch, Minerals, and Vitamins.
Lab chow diet: Cereal Grains, Meat Offal Meal, Fish Offal Meal, Whey Powder, Vegetable Oils, Soybean Protein, Cereal Offal, Corn Offal, Minerals, and Vitamins.

1.4.3 Experimental Design

Following 16 weeks of the study, the most effective treatment was chosen for further investigation in the remaining experimentation. The body weights of the five groups from week 0 to week 16 are presented in Figure 1.5 (Final average body weight after 16 weeks: LC, 27.74±0.16g; HF, 36.39±1.01g; HF+UA, 36.45±0.76g; HF+OA, 38.11±1.04g; HF+BM, 26.28±0.39g). BM treatment significantly prevented HF diet-induced body weight gain. However, UA and OA treatment failed to prevent HF diet-induced obesity. Therefore, the BM group was chosen for further investigation for the remainder of the experiments to examine the mechanisms behind why BM prevented HF diet-induced obesity. In addition, we investigated whether BM could also prevent obesity-induced associated complications including type 2 diabetes and alterations to the brain, liver, heart, and kidneys.

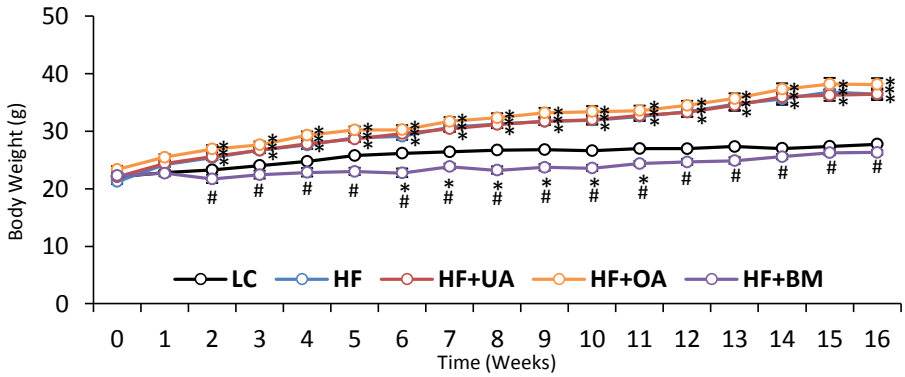

Figure 1.5. Body weights of mice fed a lab chow (LC), high-fat (HF), or HF diet supplemented with ursolic acid (UA), oleanolic acid (OA) or bardoxolone methyl (BM) (administered in drinking water at a daily dose of 10 mg/kg) from week 0 to week 16 of the experiment. Chronic administration of BM significantly prevented body weight gain in mice fed a HF diet. However, UA or OA administration failed to prevent body weight gain induced by a HF diet in mice. *, p = <0.05 vs. LC group, #, p<0.05 vs. HF group, values are means ±SEM.

To examine the effects of BM in preventing HF diet-induced alterations in hypothalamic energy balance regulation (Chapter 1), recognition memory (Chapter 2), insulin resistance and hepatic steatosis (Chapter 3), and heart and kidney pathophysiologies (Chapter 4), a chronic HF diet animal model was used before tissue was collected for analysis.

1.4.4 Euthanasia and Tissue Collection

For tissue analysis (n=14 per group), mice were euthanised at week 21 of the experiment by CO_2 infusion. Whole brains were dissected from the mice, snap frozen in liquid nitrogen and stored at -80 °C until use. Visceral and inguinal white adipose tissue (WAT) were dissected from mice and weighed. The kidneys, liver, and heart were dissected from each mouse. The full hearts and liver were weighed. The apex of the heart and a small section of the liver were cut and placed in 10% formalin. The right kidneys of each mouse were cut in half before the inferior portion was placed into 10% formalin. The remaining heart, liver and kidney tissue were snap frozen in liquid nitrogen, and stored at -80 °C until use. Blood samples were collected from the left ventricle of the heart and placed into ethylenediaminetetraacetic acid (EDTA) tubes. The plasma was then separated via centrifugation from each blood sample and stored at -80 °C.

1.4.5 Microdissection

Frozen brain sections containing the PFC and hippocampus were cut into 14 µm coronal sections with a cryostat at -18°C before being mounted on Polylysine™ microscope slides for receptor autoradiography. Further coronal brain sections were cut at 500µm before the PFC, hippocampus, mediobasal hypothalamus, and paraventricular nucleus regions were dissected for western blotting. Sections were collected using a Stoelting Brain Punch (#57401, 0.5mm diameter, Wood Dale, Stoelting Co, USA) (White, Whittington et al. 2009). The areas of the brain collected ranged from Bregma -2.34mm to -2.80mm based on a standard mouse brain atlas

(Paxinos 2002), as outlined in previous studies (Yu, Wu et al. 2013, Wu, Yu et al. 2014), Figure 1.6. Brain sections and collected tissue were stored at -20°C until use.

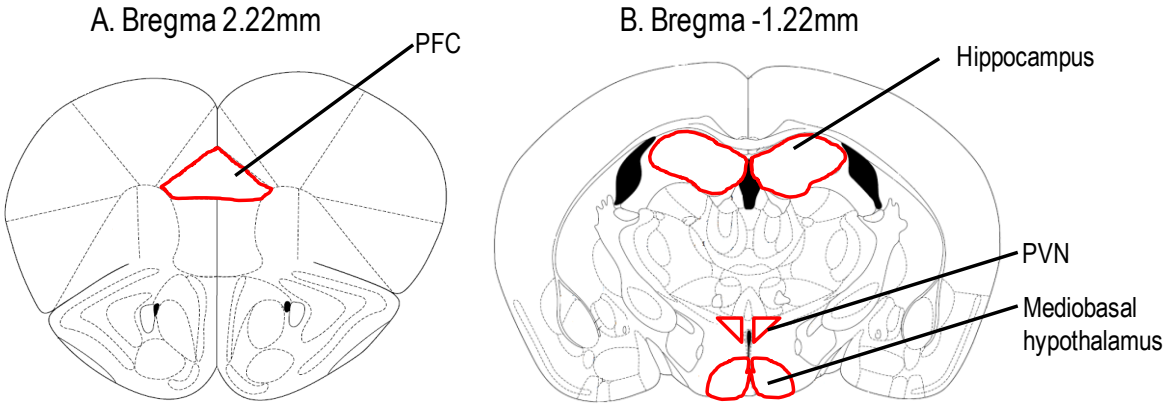

Figure 1.6. Schematic of the mouse brain. Depicts the levels of Bregma (A) 2.22mm, incorporating the prefrontal cortex (PFC); (B) -1.22mm, incorporating the hypothalamic paraventricular nucleus (PVN), mediobasal hypothalamus, and hippocampus. Modified from Paxinos and Franklin (2002). The Mouse Brain in Stereotaxic Coordinates, 2nd Ed. San Diego Academic Press, USA.

Frozen liver, heart, and kidney tissue were cut into 10 µm sections with a cryostat at -18 °C before being mounted on Polylysine™ microscope slides for histological staining. Specifically, the apex of the hearts and the superior portion of the cortex of the kidney were sectioned. The liver, left ventricle of each mouse heart and inferior portion of the kidney cortex were micro dissected from 500 µm thick frozen sections, and collected for Quantitative Real Time PCR (RT-PCR). Liver tissue was also collected from 500 µm thick frozen sections for protein analysis via western blotting. Liver, kidney and heart tissue were stored at a temperature of -80 °C until use.

1.4.6 Extraction of total, nuclear and cytosolic proteins

For total protein extraction the frozen liver, mediobasal hypothalamus, paraventricular nucleus, PFC, and hippocampus tissue samples were homogenised in homogenising buffer (containing Nonidet P-40 lysis buffer, Protease Inhibitor Cocktail, 1mM PMSF and 0.5mM β-glycerophosphate). The homogenised tissue was stored at -80 °C until use.

Nuclear and cytosolic proteins were extracted from liver tissue as described by Mobasher et al (Mobasher, Gonzalez-Rodriguez et al. 2013). Briefly, liver tissue was homogenised in a solution

containing 10Mm HEPES-KOH (pH 7.9), 10mM KCL, 1.5mM $MgCl_2$, 0.5mM DTT, 0.2mM PMSF, and protease and phosphatase inhibitors (buffer A) before incubation on ice, vortexing and centrifugation. Following centrifugation, the supernatant containing the cytosolic fraction was collected and frozen at -80 °C until use. The remaining pellet was resuspended in a solution containing 20mM HEPES-KOH (pH 7.9), 400mM NaCl, 1.5 mM $MgCl_2$ 0.2mM EDTA, 15% glycerol, 0.5mM DTT, 0.2mM PMSF and protease and phosphatase inhibitors (buffer B) before further centrifugation. Following multiple washes with buffer B and centrifugation of the pellet, the supernatant containing the nuclear fraction was collected and stored at -80 °C until use.

1.4.7 Western Blotting

The total, cytosolic, and nuclear protein concentrations were determined by a DC-Assay (Bio Rad, Hercules, USA), that was detected by an absorbance microplate reader (SpectraMax Plus 384, Molecular Devices, USA). Each sample was heated to 95°C in Laemmli buffer, before being loaded onto SDS-PAGE 4-12% gels and transferred onto ImmunoBlot™ PVDF membranes (Bio Rad, Hercules, CA, USA). The membranes were blocked with 5% BSA in TBST.

The following antibodies were incubated onto the membranes in TBST containing 1% BSA overnight at 4°C to quantify specific proteins: BDNF (sc-546), pTrkB (sc-135645), TrkB (sc-377218), pAkt (sc-135650), Akt (sc-1618), pAMPK (sc-33524), AMPK (sc-25792), pJNK (sc-6254), Nrf2 (sc-722), IL-1β (sc-7884), IL-6 (sc-7920) (Santa Cruz Biotechnology, Dallas, TX); PTP1B (#5311), pIKK (#2697), pSTAT3 (#9145), STAT3 (#4904), pFOXO1 (#9461), FOXO1 (#2880), SOCS3 (#2932), TNFα (#3707), pJAK2 (#3771) (Cell Signalling Technology, Beverly, MA). The antibodies and respective dilution factors used are presented in Table 1.2. Secondary antibodies were anti-rabbit, anti-goat or anti-mouse IgG conjugated with horseradish peroxidise (Santa Cruz Biotechnologies, USA; dilution factor 1:1000). All quantitative analyses for total and cytosolic proteins were normalised to β-actin. Nuclear proteins were normalised to Lamin B. ECL

detection reagents were used and film was exposed in the darkroom using an AGFA CP1000 Tabletop Processor (COD Medical, USA) for antibody visualisation. The bands corresponding to the proteins of interest were scanned and the band density analysed using the automatic imaging analysis system, Quantity One (Bio-Rad Laboratories, Hercules, California). Western blots were performed in triplicate for each sample; however, in some cases only two values for each sample were collected. The average of the duplicate/triplicate numbers for each sample was calculated and this number was used for statistical analysis.

Table 1.2. The antibodies used in western blotting for measuring protein expression.

Peptide/protein target	Name of Antibody	Manufacturer, catalog #, and/or name of individual providing the antibody	Species raised in; monoclonal or polyclonal	Dilution used
Akt	Akt1 Antibody (C-20)	Santa Cruz Biotechnology, sc-1618	goat polyclonal	1:200
AMPK	AMPKα1/2 Antibody (H-300)	Santa Cruz Biotechnology, sc-25792	rabbit polyclonal	1:1000
β-Actin	β-Actin (C4)	Santa Cruz Biotechnology, sc-47778	mouse monoclonal	1:1000
BDNF	BDNF Antibody (N-20)	Santa Cruz Biotechnology, sc-546	rabbit polyclonal	1:1000
FOXO1	FoxO1 (C29H4) Rabbit mAb	Cell Signalling Technology, #2880	rabbit monoclonal	1:1000
IL-1β	IL-1β Antibody (H-153)	Santa Cruz Biotechnology, sc-7884	rabbit polyclonal	1:200
IL-6	IL-6 Antibody (H-183)	Santa Cruz Biotechnology, sc-7920	rabbit polyclonal	1:200
Lamin B	Anti-Lamin B1 antibody- Nuclear Envelope marker	Abcam, ab16048	rabbit polyclonal	1:1000
Nrf2	Nrf2 Antibody (C-20)	Santa Cruz Biotechnology, sc-722	rabbit polyclonal	1:500
pAkt	p-Akt1 Antibody (Thr 308)	Santa Cruz Biotechnology, sc-135650	rabbit polyclonal	1:200
pAMPK	p-AMPKα1/2 Antibody (Thr 172)	Santa Cruz Biotechnology, sc-33524	rabbit polyclonal	1:1000
pFOXO1	Phospho-FoxO1 (Ser256)	Cell Signalling Technology, #9461	rabbit polyclonal	1:1000
pIKK	Phospho-IKKα/β (Ser176/180)	Cell Signalling Technology, #2697	rabbit monoclonal	1:1000
pJAK2	Phospho-Jak2 (Tyr1007/1008)	Cell Signalling Technology, #3771	rabbit polyclonal	1:500
pJNK	p-JNK Antibody (G-7)	Santa Cruz Biotechnology, sc-6254	mouse monoclonal	1:1000
pSTAT3	Phospho-Stat3 (Tyr705)	Cell Signalling Technology, #9145	rabbit monoclonal	1:1000
pTrkB	p-Trk B Antibody (Tyr 706)	Santa Cruz Biotechnology, sc-135645	rabbit polyclonal	1:500
PTP1B	PTP1B Antibody	Cell Signalling Technology, #5311	rabbit polyclonal	1:200
SOCS3	SOCS3 (L210) Antibody	Cell Signalling Technology, #2932	rabbit polyclonal	1:1000
STAT3	Stat3 (79D7) Rabbit mAb	Cell Signalling Technology, #4904	rabbit monoclonal	1:1000
TrkB	TrkB Antibody (F-1)	Santa Cruz Biotechnology, sc-377218	mouse monoclonal	1:500
TNFα	TNF-α Antibody	Cell Signalling Technology, #3707	rabbit polyclonal	1:1000

1.4.8 RNA extraction and RT-PCR

Total RNA was extracted from mouse liver, kidney and heart using the Aurum total RNA mini kit (Bio-Rad Laboratories, Hercules, CA) before being reversed transcribed to complimentary first strand DNA with a high-capacity cDNA reverse transcription kit (AB Applied Biosystems, California, USA) according to the manufacturer's directions. RT-PCR was performed using a Lightcycler 480 real time PCR system (F.Hoffmann-La Roche Ltd, Switzerland). A 20 ul final reaction volume containing cDNA sample and SYBR green I master mix was used to perform the experiment. Briefly, amplification was carried out with 45 cycles of 95 °C for 10 seconds, 60 °C for 30 seconds and 72 °C for 30 seconds. The expression of mRNA was normalised to an internal control, GADPH. The degree of mRNA expression was calculated using the comparative threshold cycle value (Ct) method, using the formula $2^{-\Delta\Delta Ct}$ (where ΔΔCt =ΔCt sample - ΔCt reference) as described previously (Livak and Schmittgen 2001). The primers used and gene tracking numbers are presented in Table 1.3.

Table 1.3. The primers used in RT-PCR for measuring mRNA expression.

Gene	Forward primer	Reverse primer	Gene Tracking Number (From NCBI)
IR	TTTGTCATGGATGGAGGCTA	CCTCATCTTGGGGTTGAACT	NM_010568.2
IRS-1	TCCTATCCCGAAGAGGGTCT	TGGGCATATAGCCATCATCA	NM_010570.4
G6Pase	CTGTGAGACCGGACCAGGA	GACCATAACATAGTATACACCT GCTGC	NM_008061.3
GK	GTGGTGCTTTTGAGACCCGTT	TTCAATGAAGGTGATTTCGCA	NM_010292.5
ACOX	ATGAATCCCGATCTGCGCAAG GAGC	AAAGGCATGTAACCCGTAGCA CTCC	NM_015729.3
ACC	GAAGTCAGAGCCACGGCACA	GGCAATCTCAGTTCAAGCCAGT C	NM_133360.2
SCD1	CTTCTTGCGATACACTCTGG	TGAATGTTCTTGTCGTAGGG	NM_009127.4
FAS	AGGGGTCGACCTGGTCCTCA	GCCATGCCCAGAGGGTGGTT	NM_007988.3
Nrf2	CTCGCTGGAAAAAGAAGTG	CCGTCCAGGAGTTCAGAGA	NM_010902.3
TNFα	CATCTTCTCAAAATTCGAGTGA CAA	TGGGAGTAGACAAGGTACAAC CC	NM_013693.3
IL-6	GTGGCTAAGGACCAAGACCA	GGTTTGCCGAGTAGATCTCA	NM_031168.1
IKKβ	GGCACCCTGGATGACCTAGA	CCATCTCCTGGCTGTCACCT	NM_001159774.1
IKKε	ACCACTAACTACCTGTGGCAT	ACTGCGAATAGCTTCACGATG	NM_019777.3
ET-1	GTGTCTACTTCTGCCACCTGGA CAT	GGGCTCGCACTATATAAGGGA TGAC	NM_010104.3
ET_A	CTGAAAACAATTTTTGAATTTCT TGC	TACCAAGATGTGAAGGACTGG TGG	NM_010332.2
ET_B	GTAACATGCAATCGCCCGCA	GGAACCCCAATTCCTTTAA	NM_007904.4
GADPH	TGAAGCAGGCATCTGAGGG	CGAAGGTGGAAGAGTGGGAG	NM_001289726.1

1.4.9 Immunohistochemistry

Liver, heart, and kidney sections fixed in 10% formalin were embedded in paraffin before being sectioned (5 µm) onto Polylysine™ slides. Slides were incubated overnight at 4 °C with anti-rabbit F4/80, anti-goat ET-1, anti-goat ET_B, or anti-rabbit ET_A primary antibody (1:150 Santa Cruz Biotechnology, Dallas, TX) diluted in blocking buffer. Liver sections were incubated with anti-rabbit F4/80 only. Samples were then incubated consecutively at room temperature for 30 minutes with their respective secondary antibody (1:150 Santa Cruz Biotechnology, TX) and then streptavidin-HRP polymer conjugate (1:1000 2438, Sigma-Aldrich Pty Ltd, Sydney, Australia). A DAB peroxidise substrate kit (4100, Vector Laboratories Inc, Burlingame, CA) was used for the development of the stained sections before counterstaining with H&E (POCD Scientific,

Artarmon, Australia). Three fields from three sections of each mouse were viewed under a Leica microscope and digital photographs captured. Image J software was used to quantify the area of F4/80, ET-1, ET_A, or ET_B immunoreactivity on each slide.

1.4.10 Haematoxylin and Eosin (H&E) staining

Frozen liver, kidney and heart sections (10 μm) were stained with Haematoxylin and Eosin (POCD Scientific, Artamon, Australia) for 30 seconds each. Three fields from three sections of each mouse were viewed under a Leica microscope and digital photographs were captured in order to analyse histological parameters for each tissue.

For the liver tissue, steatosis and ballooning were scored according to the method described by Kleiner and colleagues (Kleiner, Brunt et al. 2005). The steatosis grades were as follows: 0, <5%; 1, 5%–33%; 2, >33%–66%; 3, >66%. The ballooning classifications were grouped as: 0, no ballooning cells; 1, few ballooning cells; 2, many cells/prominent ballooning. Glomerular and Bowman's capsule hypertrophy in the kidneys were calculated according to the methods described by previous studies (Al-Douahji, Brugarolas et al. 1999, Henegar, Bigler et al. 2001). In the heart tissue, myocytes were measured quantitatively using the software, Image J according to our previous study (Dinh, Szabo et al. 2015).

1.4.11 Oil Red O staining

Oil Red O staining was used to examine lipid accumulation in the liver, heart, and kidneys, as described previously (Kudo, Tamagawa et al. 2007). Briefly, frozen liver, heart, and kidney sections (10 μm) were stained with 0.5% Oil Red O (Sigma-Aldrich) for 15 minutes and then washed. Three fields from three sections collected from each mouse were viewed under a Leica microscope, and digital photographs were captured. Image J software (http://imagej.nih.gov/ij/download.html) was used to quantify the staining, which corresponds to the percentage of stained lipid droplets on an area of each slide (Mehlem, Hagberg et al. 2013).

1.4.12 Statistical Analysis

A power calculation analysis was performed in experimental design and revealed a power of 80-85% (JMP 5.1, SAS Institute Inc, USA), based on previous studies (Zhao, Sim et al. 2005). In order to achieve a power of 80% in this project, a minimum of 12 animals per group were required to examine body weight, food intake, and recognition memory, whereas a minimum of 6 animals per group were required for further histological and biochemical analysis in order for results to be significantly different at an alpha level of 0.05.

Data were analysed using the statistical package SPSS 20 (SPSS, Chicago, IL). Data was first tested for normality using Kolmogorov-Smirnov Tests, before differences between mice fed a LC, HF, and HF supplemented with BM diet were determined by one-way analysis of variance (ANOVA). This was followed by the post hoc Tukey-Kramer honestly significant difference (HSD) test for multiple comparisons among the groups. Pearson's correlations were used to examine the relationship between recognition index and BDNF levels, recognition index and NMDA receptor density, and AMPK phosphorylation and BDNF levels in the PFC and hippocampus. All data are expressed as mean ± SEM. A *p* value less than 0.05 was considered statistically significant.

1.5 OVERVIEW OF THESIS

The worldwide increase in rates of obesity is largely driving an increase in type 2 diabetes and associated complications affecting vital organs including the brain, liver, heart, and kidneys. Therefore, there is an urgency to find novel therapeutics that have the ability to prevent obesity, type 2 diabetes, and the development of associated complications. I decided to search for potential therapeutics by looking at natural compounds and their derivatives. After careful consideration, I decided to test the effects of the pentacyclic triterpenes, OA, UA, and a derivative, BM, in mice fed a chronic HF diet and to choose the most potent compound for subsequent experimentation. From preliminary testing, it was uncovered that BM, a highly potent OA derivative, had the ability to prevent body weight gain in mice fed a chronic HF diet. However, UA and OA treatment had no effect in preventing HF diet-induced body weight gain. From this, the molecular mechanisms underlying the ability of BM to prevent HF diet-induced obesity were examined by investigating energy balance signalling in the hypothalamus. In addition, whether BM administration could also prevent the development of obesity-induced insulin resistance, memory deficits, hepatic steatosis, and cardiorenal diseases was also determined. Summary abstracts from each study are presented in the following sections (1.5.1-1.5.4).

1.5.1 BM prevents body weight gain, hypothalamic inflammation, and leptin resistance in male mice fed a HF diet

Neuroregulation of negative energy balance is largely controlled by the mediobasal and paraventricular nuclei regions of the hypothalamus via leptin signal transduction. HF diet-induced obesity is associated with hypothalamic leptin resistance and low grade chronic inflammation, which largely impairs this neuroregulation of negative energy balance. Recently, BM has been shown to have anti-inflammatory effects. The hypothesis that BM would prevent diet-induced obesity, leptin resistance and inflammation in mice fed a HF diet was tested. Oral administration

of BM via drinking water (10 mg/kg daily) for 21 weeks significantly prevented an increase in food intake, body weight, hyperleptinemia, and peripheral fat accumulation in mice fed a HF diet. Furthermore, BM treatment prevented decreased the anorexigenic effects of peripheral leptin administration induced by a HF diet. In the mediobasal and paraventricular nuclei regions of the hypothalamus, BM administration prevented HF diet-induced impairments of downstream leptin JAK2-Akt-FOXO1 signalling. BM treatment also prevented an increase in the inflammatory mediator, pJNK, and cytokines, TNFα and IL-6, in these two hypothalamic regions. These results identify a potential novel neuropharmacological application for BM to prevent HF diet-induced obesity, hypothalamic inflammation, and leptin resistance.

1.5.2 BM prevents HF diet-induced alterations in prefrontal cortex signalling molecules involved in recognition memory

HF diets are known to induce changes in synaptic plasticity in the forebrain leading to learning and memory impairments. Previous studies of OA derivatives have found that these compounds can cross the blood brain barrier to prevent neuronal cell death. The hypothesis that BM would prevent diet-induced cognitive deficits in mice fed a HF diet was examined. C57BL/6J male mice were fed a LC (5% of energy as fat), HF (40% of energy as fat), or HF diet supplemented with 10 mg/kg/day BM orally for 21 weeks. Recognition memory was assessed by performing a novel object recognition test on the treated mice. Downstream BDNF signalling molecules were examined in the PFC and hippocampus of mice via western blotting and NMDA receptor binding. BM treatment prevented HF diet- induced impairment in recognition memory. In HF diet-fed mice, BM administration attenuated alterations in NMDA receptor binding density in the PFC; however, no changes were seen in the hippocampus. In the PFC and hippocampus of HF diet-fed mice, BM administration improved downstream BDNF signalling as indicated by increased protein levels of BDNF, pTrkB and pAkt, and increased pAMPK. BM administration also prevented HF

diet-induced increase in the protein levels of inflammatory molecules, pJNK in the PFC, and PTP1B in both the PFC and hippocampus. In summary, these findings suggest that BM prevents HF diet-induced impairments in recognition memory by improving downstream BDNF signal transduction, increasing pAMPK, and reducing inflammation in the PFC and hippocampus.

1.5.3 BM prevents insulin resistance and the development of hepatic steatosis in mice fed a HF diet.

HF diet-induced obesity is a major risk factor for the development of insulin resistance and hepatic steatosis. The hypothesis that BM would prevent the development of insulin resistance and hepatic steatosis in mice fed a HF diet was determined. C57BL/6J male mice were fed a LC, HF (40% fat), or HF diet supplemented with 10 mg/kg/day BM orally for 21 weeks. Glucose metabolism was assessed using a GTT and IST. Signalling molecules involved in insulin resistance, inflammation, and lipid metabolism were examined in liver tissue via western blotting and RT-PCR. BM prevented HF diet-induced insulin resistance and alterations in the protein levels of PTP1B, FOXO1 and BDNF, and expression of the *IR*, *IRS-1* and *G6Pase* genes. Furthermore, BM prevented fat accumulation in the liver and decreases in the β-oxidation gene, *ACOX,* in mice fed a HF diet. In the livers of HF fed mice, BM administration prevented HF diet-induced macrophage infiltration, inflammation as indicated by reduced IL-6 and STAT3 protein levels and *TNFα* mRNA expression, and increased *Nrf2* mRNA expression and nuclear protein levels. These findings suggest that BM prevents HF diet-induced insulin resistance and the development of hepatic steatosis in mice fed a chronic HF diet through modulation of molecules involved in insulin signalling, lipid metabolism and inflammation in the liver.

1.5.4 BM prevents the development and progression of cardiac and renal pathophysiologies in mice fed a HF diet

Obesity is a major risk factor for the development of associated complications, such as heart and kidney failure. BM was administrated to mice fed a HF diet for 21 weeks to determine if it would prevent the development of obesity-associated cardiac and renal pathophysiologies. Histological analysis revealed that BM prevented HF diet-induced development of structural changes in the heart and kidneys. BM prevented HF diet-induced decreases in myocyte number in cardiac tissue and renal corpuscle hypertrophy in the kidney. Furthermore, in both the hearts and kidneys of mice fed a HF diet, BM administration prevented HF diet-induced increases in fat accumulation, macrophage infiltration and *TNFα* gene expression. These findings suggest that BM prevents HF diet-induced developments of cardiac and renal pathophysiologies in mice fed a chronic HF diet, by preventing inflammation. Moreover, these results suggest that BM has the potential as a novel therapeutic for preventing obesity-induced cardiac and renal pathophysiologies.

1.5.5 Summary

In conclusion, obesity was successfully modelled in male C57BL/6 mice following a HF diet for 21 weeks. Furthermore, mice fed a HF diet developed obesity-associated complications including leptin resistance, insulin resistance, cognitive deficits, and liver, kidney, and heart pathophysiologies. Diet-induced obesity and these associated co-morbidities were prevented by oral administration of BM in drinking water at a dosage of 10 mg/kg in mice fed a HF diet for 21 weeks. Results from the present study suggest that BM prevented the development of HF diet-induced obesity and associated type 2 diabetes and recognition memory deficits by targeting hypothalamic leptin signalling, hepatic insulin signalling, and downstream BDNF signalling in the forebrain respectively. Moreover, the ability of BM to prevent obesity-induced peripheral tissue damage of the liver, heart, and kidneys was suggested to be as a result of its potent anti-

inflammatory mechanisms. Overall, these findings highlight BM as a potential novel therapeutic in preventing the development and progression of HF diet-induced obesity and associated type 2 diabetes, cognitive deficits, and pathophysiologies of the liver, heart, and kidneys.

Chapter Two

Bardoxolone methyl prevents body weight gain, hypothalamic inflammation, and leptin resistance in male mice fed a high-fat diet

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2.1 Author Contributions

D.Camer was a designer of this study, performed all of the experiments, analysed all the data, and wrote the first draft of the manuscript, which all authors reviewed and approved for publication.

2.2 Collaborator Statement

We hereby declare that the statement in section 2.1 pertaining to the contributions of D.Camer is correct.

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Bardoxolone methyl prevents body weight gain, hypothalamic inflammation, and leptin resistance in male mice fed a high-fat diet

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Abstract

Neuroregulation of negative energy balance is largely controlled by the mediobasal and paraventricular nuclei regions of the hypothalamus via leptin signal transduction. High-fat (HF) diet-induced obesity is associated with hypothalamic leptin resistance and low grade chronic inflammation, which largely impairs this neuroregulation of negative energy balance. Recently, a derivative of oleanolic acid, bardoxolone methyl (BM) has been shown to have anti-inflammatory effects. We tested the hypothesis that BM would prevent diet-induced obesity, leptin resistance and inflammation in mice fed a HF diet. Oral administration of BM via drinking water (10 mg/kg daily) for 21 weeks significantly prevented an increase in body weight, energy intake, hyperleptinemia and peripheral fat accumulation in mice fed a HF diet. Furthermore, BM treatment prevented decreased anorexigenic effects of peripheral leptin administration induced by a HF diet. In the mediobasal and paraventricular nuclei regions of the hypothalamus, BM administration prevented HF diet-induced impairments of downstream leptin JAK2-Akt-FOXO1 signalling. BM treatment also prevented an increase in the inflammatory mediator, pJNK and cytokines, TNFα and IL-6 in these two hypothalamic regions. These results identify a potential novel neuropharmacological application for BM to prevent HF diet-induced obesity, hypothalamic inflammation and leptin resistance.

1. Introduction

Obesity is currently a major health problem characterised by a deregulation of energy balance that is attributed to an increase in consumption of palatable high-fat (HF) food and reduced energy expenditure. The prevalence of obesity is a growing problem since it greatly increases the risk of developing associated complications such as type 2 diabetes and cardiovascular disease (Kahn, Hull et al. 2006, Forouhi and Wareham 2010). There is compelling evidence that overnutrition and subsequent obesity leads to chronic inflammation and leptin resistance in the hypothalamus, an area of the brain that plays a critical role in maintaining energy homeostasis. Therefore, the hypothalamus appears a promising target of future novel therapeutics for preventing the development of obesity and associated pathophysiologies.

It is well established that hypothalamic inflammation is a key characteristic of obesity in rodents and humans (Cai and Liu 2011). Recent research has demonstrated that a HF diet results in low grade hypothalamic inflammation in rodents (Thaler, Yi et al. 2012). Furthermore, within a week of starting a HF diet, rodents have increased mRNA expression of the proinflammatory cytokines tumour necrosis factor alpha (TNFα) and interleukin 6 (IL-6) in the hypothalamus (Thaler, Yi et al. 2012). Hypothalamic inflammation leads to the development of central leptin resistance through activation of protein tyrosine phosphatase 1B (PTP1B), an inhibitor of leptin signalling (Zhang, Zhang et al. 2008, Milanski, Arruda et al. 2012). In the hypothalamus, leptin binds to long form leptin receptors and functions to regulate food intake and energy expenditure via neuronal interactions known as central leptin signalling (Friedman and Halaas 1998, Elmquist, Elias et al. 1999, Bates and Myers 2003). In a normal functioning state, central leptin signalling allows the suppression of hunger signals causing satiety (Friedman and Halaas 1998, Elmquist, Elias et al. 1999, Bates and Myers 2003). Obesity from a HF diet has been demonstrated in both rodents and humans to cause central leptin resistance which limits the clinical effectiveness of exogenous leptin administration (Caro, Kolaczynski et al. 1996, Nam, Kratzsch et al. 2001). Central leptin

resistance has been suggested to occur since it has been found that although leptin levels in the cerebrospinal fluid (CSF) is 30% higher in obese individuals than individuals with a lean body mass, leptin downstream orexigenic neuropeptide, neuropeptide Y (NPY) are not reduced in the CSF (Caro, Kolaczynski et al. 1996, Nam, Kratzsch et al. 2001). Therefore, novel therapeutics that target hypothalamic inflammation and leptin resistance has the potential to prevent the development of obesity and associated complications such as type 2 diabetes.

Oleanolic acid (OA) is a natural compound that has shown a number of therapeutic benefits in the treatment and prevention of obesity and associated complications such as type 2 diabetes (Camer, Yu et al. 2014). In several *in vitro* studies, OA has been found to improve leptin signalling by activating the phosphorylation of protein kinase b (Akt) and reducing the leptin signalling inhibitor, PTP1B (Na, Oh et al. 2006, Jung, Ha et al. 2007, Lin, Zhang et al. 2008, Ramirez-Espinosa, Rios et al. 2011). However, derivatives of OA have been found to be significantly more potent and have a higher bioavailability than in their natural form (Zhang, Zhang et al. 2008). An example of a highly potent OA synthetic derivative is bardoxolone methyl (BM), which has attracted wide attention due to its anti-inflammatory effects (Ahmad, Raina et al. 2006, Wang, Garvin et al. 2011, Liby and Sporn 2012, Reisman, Chertow et al. 2012). BM has completed phase II of human clinical trials for treating chronic kidney disease (CKD) in individuals with type 2 diabetes (Pergola, Raskin et al. 2011, NIH 2012). Interestingly one of the side effects, body weight loss, has been reported in the phase II human clinical trials in a population with CKD and type 2 diabetes (Pergola, Raskin et al. 2011, NIH 2012). However, the effects of BM on the hypothalamus have previously been unexplored. Therefore, in this study, we investigated whether chronic treatment with BM could prevent body weight gain and the development of hypothalamic inflammation and leptin resistance in mice fed a HF diet.

2. Materials and Methods

2.1 HF diet-induced obesity animal model

12 week old male C57BL/6J mice were purchased from the Animal Resource Centre (Perth, Western Australia) and maintained in the animal facility at the University of Wollongong. The procedures were undertaken in accordance with the *Australian Code of Practice for the Care and Use of Animals for Scientific Purposes* and were approved by the Animal Ethics Committee, University of Wollongong, Wollongong, Australia (AE 12/15). Mice were housed individually in environmentally controlled conditions (temperature 22 °C, 12hr light/dark cycle). Following 1 week of acclimatisation, mice were randomly divided into 3 groups (n=14 per group). For the next 21 weeks, one group of mice were fed a lab chow (LC) diet (5% of energy as fat; Vella Stock Feeds, Doonside, New South Wales, Australia), while the other two groups were fed a HF diet (40% of energy as fat; SF11-095, Specialty Feeds, Glen Forrest, Western Australia). One of the groups of mice fed the HF diet were also given an oral daily dose of BM (10 mg/kg) administered in their drinking water for the duration of the study. This dosage was chosen as per our previous studies (Camer, Yu et al. 2015, Camer, Yu et al. 2015). Body weight and energy intake were measured weekly.

2.2 Peripheral leptin sensitivity test

At week 16 of the study, each group of mice (n=14 per group) were further divided into leptin treated or saline groups (n=7 per group). Following overnight fasting, mice were administered with an intraperitoneal (i.p) leptin or saline injection at a dosage of 2μg/g body weight. Food intake was measured every 1, 4 and 24 hours and body weight was measured 24 and 48 hours following leptin or saline injection as reported previously (Lin, Thomas et al. 2000).

2.3 Tissue collection and sample preparations

Mice were euthanised at week 21 of the experiment (n=14). Visceral and inguinal white adipose tissue (WAT) were dissected from mice and weighed. Brains were collected and stored at -80 °C for further analyses as detailed below.

2.4 Microdissection

500μm frozen brain sections were cut using a cryostat, at a temperature of -18°C, at levels ranging from Bregma -1.22mm to -2.72mm based on a standard mouse brain atlas (Paxinos 2002) as outlined in our previous studies (Yu, Wu et al. 2013, Wu, Yu et al. 2014). The mediobasal and paraventricular nucleus regions of the hypothalamus were dissected and collected using a Stoelting Brain Punch (#57401, 0.5mm diameter, Wood Dale, Stoelting Co, USA) (White, Whittington et al. 2009).

2.5 Western Blot analysis

For protein extraction the frozen mediobasal and paraventricular nucleus regions of the hypothalamus were homogenised in Nonidet P-40 lysis buffer. The following antibodies were used to quantify specific proteins: BDNF (sc-546), pTrkB (sc-135645), pAkt (sc-135650), pAMPK (sc-33524), pJNK (sc-6254), IL-6 (sc-7920) (Santa Cruz Biotechnology, Dallas, TX); PTP1B (#5311), TNFα (#3707), pSTAT3 (#9145), STAT3 (#4904), pFOXO1 (#9461), FOXO1 (#2880), pJAK2 (#3771) (Cell Signalling Technology, Beverly, MA). The bands corresponding to the proteins of interest were scanned before the band densities were analysed using Quantity One software (Bio-Rad Laboratories, Hercules, California). All quantitative analyses were normalised to β-actin.

2.6 Luminex Assay

Blood was collected in EDTA tubes from mice following euthanasia. Following centrifugation, plasma was extracted, collected and stored at -80 °C. Plasma leptin levels were measured using luminex assay kits according to manufacturer guidelines (Bio-Rad Diabetes Kit, Sydney).

2.7 Statistics

Data were analysed using the statistical software package SPSS 20 (SPSS, Chicago, IL). All data were first tested for normality using a Kolmogorov-Smirnov normality test. Differences between mice fed a LC, HF, and HF diet with BM treatment were determined by one-way ANOVA. This was followed by the post hoc Tukey-Kramer honestly significant difference (HSD) test for multiple comparisons among the groups. A p value of <0.05 was considered statistically significant. Values are expressed as the mean ± SEM.

3. Results

3.1 Bardoxolone methyl prevented body weight gain, an increase in energy intake and accumulation of adipose tissue in mice fed a high-fat diet

Mice were fed a HF diet for 21 weeks and weighed weekly to assess body weight gain. Body weight steadily increased in HF diet fed mice compared with LC diet fed mice (Figure 1A). However, oral administration of BM significantly prevented body weight gain during the 21 week treatment period, with significance first achieved at week 2, in mice fed a HF diet (Figure 1A). Furthermore, from weeks 6 to 11 the BM treated group had a significantly lower body weight compared to the LC diet fed group. The final body weight of BM treated animals was significantly lower than the HF diet fed group after 21 weeks of treatment (Final body weight: -31.12%, p = <0.001, Figure 1A). There were no significant differences in final body weight between the BM treatment group and LC group.

Overall, BM administration significantly prevented HF diet-induced increases in energy intake by 12.72% (Average 24 hour energy intake: LC= 17.3±0.5 kcal, HF= 20.0±0.5 kcal, HF+BM= 17.5±0.5 kcal, p= <0.05), and a reduction in energy intake was observed in weeks 5, 7, 8, 9,11,12,19 and 20 of the study (Figure 1B). In addition, average 24 hour energy efficiency (body weight gain/energy intake) was significantly lower in BM treated mice compared to HF diet fed mice by 61.80% (Average 24 hour energy efficiency: LC= 0.018±0.06 g/kcal, HF= 0.042±0.01 g/kcal, HF+BM= 0.016±0.01 g/kcal, p= <0.05). Consistent with reduced body weight gain, BM also significantly prevented body fat accumulation in mice fed a HF diet compared with the control HF diet group (Figure 1C and D). Compared with mice fed a HF diet only, BM treated mice had significantly lower amounts of epididymal, perirenal, and inguinal fat deposits (Epididymal fat weight -69.05%; perirenal fat weight -72.36%; inguinal fat weight -67.17 %; all p = <0.001, Table 1). Taken together, these results suggest that BM's ability to reduce body weight gain and fat accumulation may be through reducing energy intake.

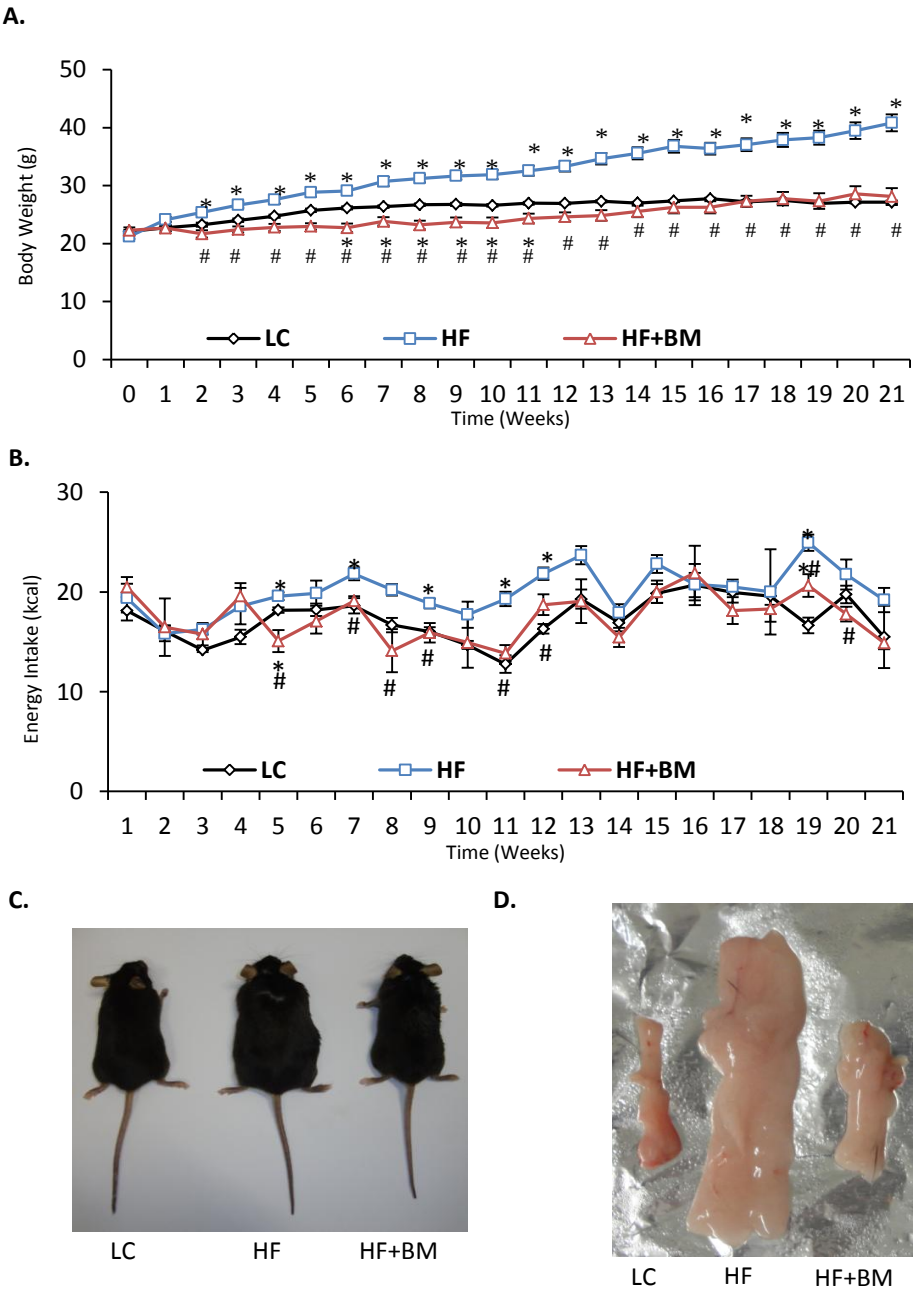

Figure 1. Effect of chronic administration of bardoxolone methyl (BM) on body weight gain, energy intake and peripheral fat accumulation in mice fed a high-fat (HF) diet for 21 weeks (n= 14 per group). Chronic administration of BM (10 mg/kg in drinking water for 21 weeks) significantly prevented body weight gain (A) and energy intake (B) resulting in a reduced body size (C) and peripheral fat accumulation (D). *, p = <0.05 vs. lab chow (LC) group, #, p<0.05 vs. HF group, values are means ±SEM.

Table 1 Weight of fat deposits in mice following 21 weeks of LC, HF or HF + BM diet

Weight (g)	LC	HF	HF+BM	F value	P value
Epididymal fat	0.24±0.04[b]	1.64±0.19[a]	0.51±0.08[b]	36.027	<0.001
Perirenal fat	0.07±0.00[b]	1.02±0.08[a]	0.28±0.05[b]	78.695	<0.001
Inguinal fat	0.38±0.05[b]	1.72±0.16[a]	0.57±0.05[b]	50.797	<0.001

Values are means ±SEM. LC, lab chow diet, HF, high-fat diet, HF+BM, high-fat diet and bardoxolone methyl treatment. [a]*P*<0.05 vs. LC, [b]*P*<0.05 vs. HF.

3.2 Bardoxolone methyl prevented leptin resistance and hyperleptinemia in mice fed a high-fat diet

Hyperleptinemia and leptin resistance are characteristic features of obesity (Bodkin, Nicolson et al. 1996, Dagogo-Jack, Fanelli et al. 1996). At week 16 of the experiment, a peripheral leptin sensitivity test was performed to determine if BM could prevent leptin insensitivity in mice fed a HF diet. LC fed mice had significantly reduced body weight at 0-24 hours and 24-48 hours, and reduced energy intake 4-24 hours following i.p leptin administration compared to saline injected controls (Figures 2A and B). In comparison, HF diet-fed mice demonstrated no changes in body weight or energy intake following leptin administration compared to saline injection (Figures 2A and B), suggesting that they were insensitive to leptin. However, BM treatment in mice fed a HF diet significantly prevented leptin insensitivity as indicated by a reduction of body weight at 0-24 hours and energy intake 4-24 hours in mice injected with leptin compared with saline injection (Figure 2A and B). However, this reduction of body weight did not last 24-48 hours later following leptin injection in BM treated mice (Figure 2A). In addition, there were no significant differences in energy intake between any of the groups at 0-1 hour, and 1-4 hours following leptin or saline administration (Figure 2B). In line with these results, mice fed a HF diet for 21 weeks had significantly elevated plasma leptin levels compared to the LC group (Figures 2C). This elevation in plasma leptin levels was significantly prevented by BM treatment (Figures 2C). These results suggest that BM can prevent HF diet induced leptin resistance and hyperleptinemia.

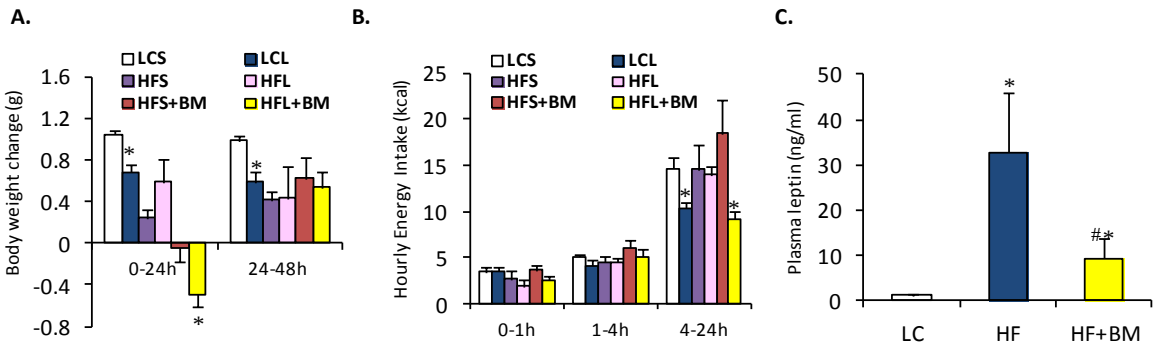

Figure 2. Effect of chronic bardoxolone methyl (BM) levels on peripheral leptin sensitivity and plasma leptin levels in mice fed a high-fat (HF) diet (n=7 per group). Chronic administration of bardoxolone methyl (BM; 10 mg/kg in drinking water) significantly prevented HF diet-induced leptin resistance as demonstrated by reduced 24 hour body weight (A) and energy intake (B) following intraperitoneal (i.p) leptin injection in mice fed a HF diet for 16 weeks. *, p = <.0.05 vs. saline injection. BM treatment also prevented plasma hyperleptinemia (C) in mice fed a HF diet for 21 weeks. *, p = <.0.05 vs. lab chow (LC) group, #, p = <.0.05 vs. HF group, values are means ±SEM.

3.3 Bardoxolone methyl prevented high-fat diet-induced alterations in energy balance regulating molecules in the mediobasal and paraventricular nucleus regions of the hypothalamus

We evaluated the effect of BM on the expression of energy balance regulatory molecules in the mediobasal and paraventricular nucleus regions of the hypothalamus of HF diet fed mice using western blotting. In both regions of the hypothalamus tested, western blot analysis showed that a HF diet significantly reduced brain-derived neurotrophic factor (BDNF) levels and the phosphorylation of janus kinase 2 (JAK2), Akt, and forkhead box protein O1 (FOXO1), which was significantly reversed by BM treatment (p = <0.05, Figures 3 and 4). Furthermore, HF diet induced increases in signal transducer and activator of transcription 3 (STAT3), PTP1B, and the phosphorylation of AMP-activated protein kinase (AMPK), was significantly prevented by BM administration (p = <0.05, Figures 3 and 4). In addition, in the paraventricular nucleus region of the hypothalamus FOXO1 protein levels were significantly elevated in HF diet fed mice (p = <0.05, Figure 4A). This increase in FOXO1 levels in the paraventricular nucleus was significantly prevented by BM treatment (p = <0.05, Figure 4A). However, in the mediobasal region of the hypothalamus, there were no significant differences in FOXO1 protein levels between the groups (p = >0.05, Figure 3A). There were also no significant differences in the phosphorylation of

STAT3 and tropomyosin receptor kinase B (TrkB) between any of the groups in either of the regions of the hypothalamus (p = >0.05, Figures 3B, 3D, 4B and 4D). These results suggest that BM prevents HF diet-induced decreases in the negative energy balance associated molecules, BDNF, pJAK2, pAkt, and pFOXO1, and increases in the positive energy balance associated molecules, STAT3, PTP1B and pAMPK, in both the mediobasal and paraventricular nucleus regions of the hypothalamus. Furthermore, these results suggest that BM prevents HF diet-induced elevations in FOXO1 levels in the paraventricular region of the hypothalamus.

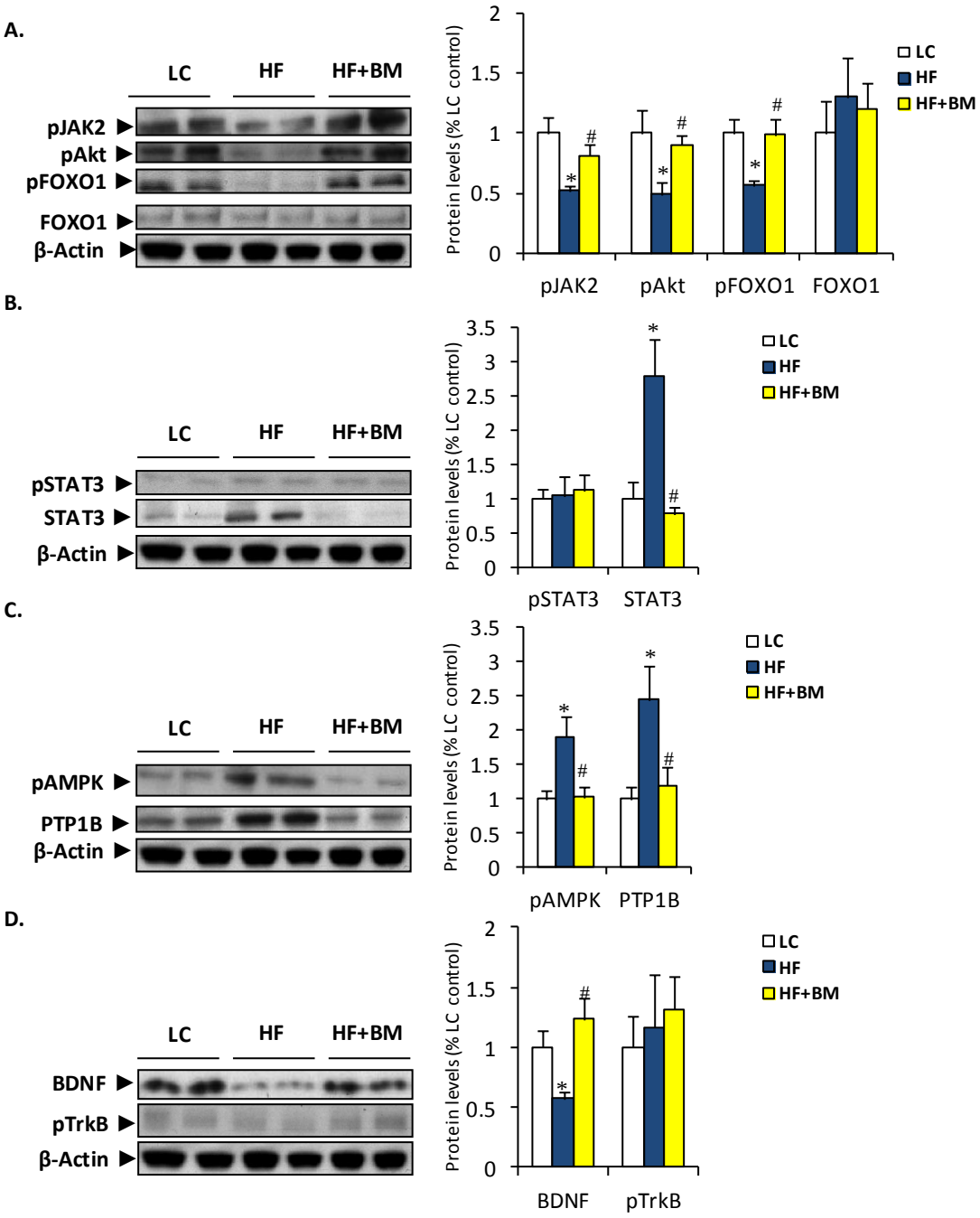

Figure 3. Effect of chronic administration of bardoxolone methyl (BM) treatment on key signalling molecules involved in energy balance in the mediobasal region of the hypothalamus in mice fed a HF diet for 21 weeks (n= 6-7 per group). Chronic treatment of BM significantly prevented high-fat (HF) diet-induced alterations in (A) downstream pJAK2-Akt-FOXO1 leptin signalling molecules, (B) STAT3, (C) negative regulators and (D) BDNF signalling molecules. *, p = <0.05 vs. lab chow (LC) group, #, p = <0.05 vs. HF group, values are means ±SEM.

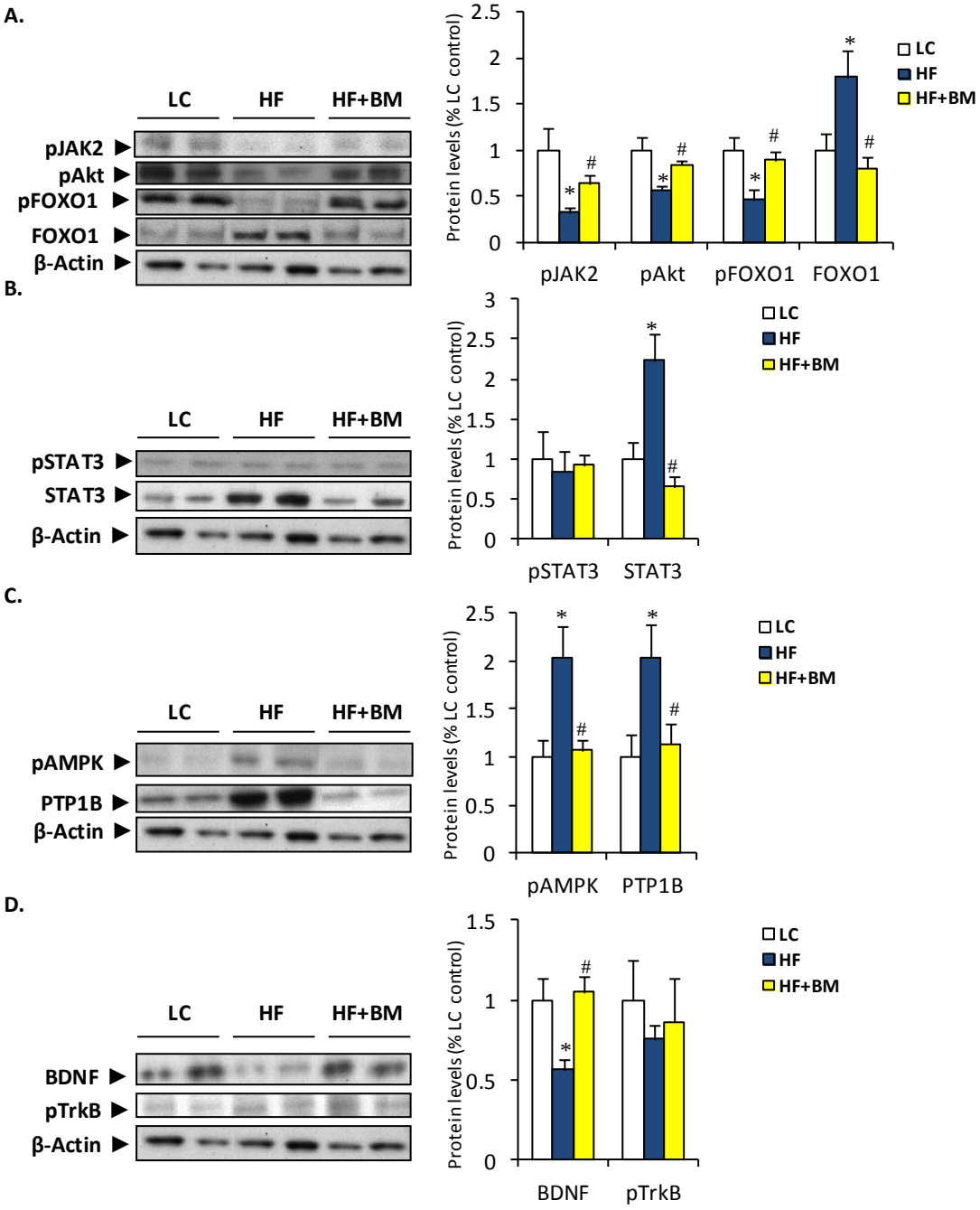

Figure 4. Effect of chronic administration of bardoxolone methyl (BM) treatment on key signalling molecules involved in energy balance in the paraventricular nuclei region of the hypothalamus in mice fed a HF diet for 21 weeks (n= 6-7 per group). Chronic treatment of BM significantly prevented high-fat (HF) diet-induced alterations in (A) downstream pJAK2-Akt-FOXO1 leptin signalling molecules, (B) STAT3, (C) negative regulators and (D) BDNF signalling molecules. *, p = <0.05 vs. lab chow (LC) group, #, p = <0.05 vs. HF group, values are means ±SEM.

3.4 Bardoxolone methyl prevented high-fat diet-induced inflammation in the mediobasal and paraventricular nucleus regions of the hypothalamus

The phosphorylation of the downstream inflammatory mediator, c-Jun N-terminal kinase (JNK), and protein expression of the cytokines, TNFα and IL-6, were measured using western blotting in the mediobasal and paraventricular nucleus of mice fed a HF diet in order to determine if BM treatment could prevent hypothalamic neuroinflammation. In HF group, protein phosphorylation of JNK and the expression of TNFα and IL-6 were significantly increased in both regions of the hypothalamus (p = <0.05, Figures 5A and B). These HF diet-induced elevations in inflammation found in the mediobasal and paraventricular nucleus regions of the hypothalamus were significantly prevented by BM administration (p = <0.05, Figures 5A and B). These results suggest that BM treatment prevented a HF diet-induced inflammatory response in the mediobasal and paraventricular nucleus regions of the hypothalamus.

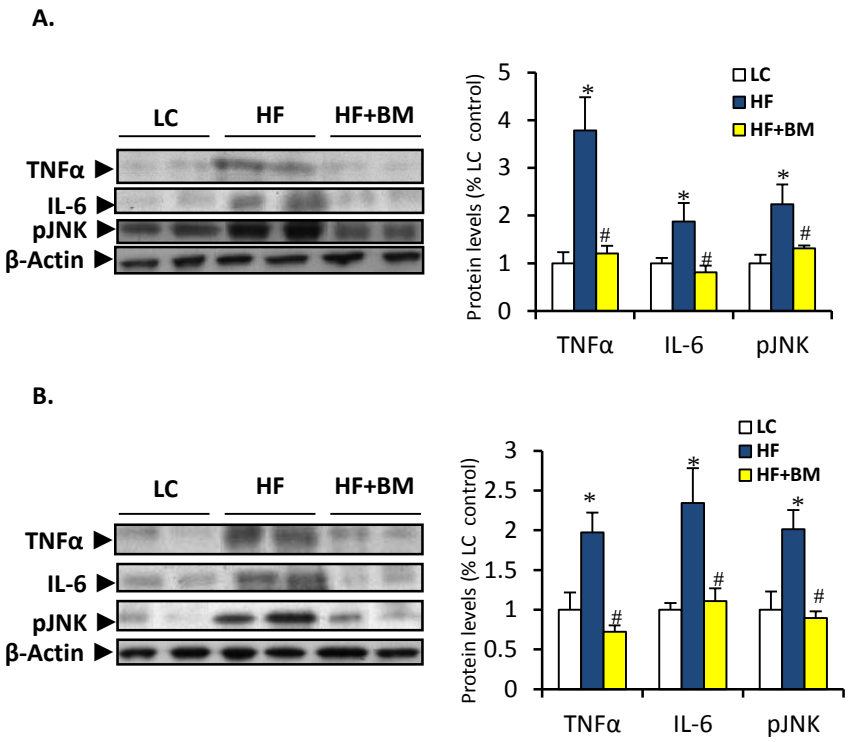

Figure 5. Effect of chronic administration of bardoxolone methyl (BM) treatment on protein levels of inflammatory mediators in the hypothalamus of mice fed a high-fat (HF) diet for 21 weeks (n=6-7 per group). Chronic treatment of BM significantly prevented HF diet-induced increase in phosphorylation of JNK, and TNFα and IL-6 levels in the mediobasal (A) and paraventricular nuclei (B) regions of the hypothalamus in mice. *, p = <0.05 vs. lab chow (LC) group, #, p = <0.05 vs. HF group, values are means ±SEM.

4. Discussion

This study demonstrated that BM treatment prevented HF diet-induced obesity, increased energy intake, peripheral fat accumulation, hypothalamic inflammation and leptin resistance in HF diet fed mice. BM treatment significantly prevented elevated proinflammatory signalling molecules in both the mediobasal and paraventricular nuclei regions of the hypothalamus. The prevention of HF diet-induced hypothalamic inflammation by BM administration was coupled with the prevention of leptin resistance in the treated animals. This was evidenced by the prevention of HF diet-induced impairments in hypothalamic leptin signalling molecules. Furthermore, BM administration prevented HF diet-induced impairments to the anorexigenic effects of peripheral leptin administration, as demonstrated by its ability to reduce energy intake and body weight 24 hours following a leptin injection.

In a phase II human clinical trial of patients with chronic kidney disease and type 2 diabetes treated with BM, dramatic weight loss was observed where treated patients lost 5-10kg more than the placebo group over a 24 week period (Pergola, Raskin et al. 2011). However, the possible mechanisms explaining this effect were unclear. The current study is the first to demonstrate BM's effect in a chronic HF diet-induced obesity prevention study. Our results demonstrated that BM treatment in mice fed a HF diet for 21 weeks significantly prevented body weight gain and fat accumulation. Furthermore, similarly to Parekh and colleagues (Parekh, Petro et al. 1998), we found that energy efficiency was significantly increased in the HF diet group which was prevented by BM administration. In addition, BM administration prevented HF diet-induced increases in energy intake, suggesting that BM regulates negative energy balance by targeting the hypothalamus, an important region for maintaining energy homeostasis.

In the hypothalamus of the brain, leptin plays a crucial role in the control of energy intake. In most obese individuals, this control of energy intake by leptin is impaired resulting in leptin resistance. In this study we confirmed that mice fed a chronic HF diet were leptin resistant. An i.p injection of leptin significantly decreased energy intake and body weight 24 hours following the injection in mice fed a LC diet but not in HF diet fed mice. In addition, HF diet fed mice had significantly higher plasma leptin levels than LC fed mice. One mechanism that can explain the development of leptin resistance is hyperleptinemia in diet-induced obese mice (Knight, Hannan et al. 2010). In the current study, BM significantly prevented HF diet-induced leptin resistance and hyperleptinemia as evidenced by restored leptin in reducing body weight and energy intake 24 hours following i.p leptin injection, and significantly improved hyperleptinemia. BM administration may have prevented HF diet-induced overstimulation and subsequent desensitisation of the leptin receptor and downstream signalling, thereby preventing leptin resistance.

Hypothalamic leptin-JAK2-Akt-FOXO1 signalling is essential for the regulation of energy balance (Munzberg and Myers 2005). The mediobasal and paraventricular nuclei regions of the hypothalamus are important areas in controlling energy balance through central leptin signalling (Bell, Bhatnagar et al. 2000). Central leptin administration via intracerebroventricular (i.c.v) injection results in an increase of JAK2 and Akt phosphorylation (Roman, Reis et al. 2010). However, an i.c.v central leptin injection results in an increase in the phosphorylation of Akt in lean mice but not obese mice, which suggests that central leptin resistance may be caused by a deregulation in phosphorylated Akt and leptin signalling (Metlakunta, Sahu et al. 2008). Phosphorylated Akt (pAkt) can subsequently phosphorylate and inactivate FOXO1, a transcription factor in the hypothalamus (Kim, Pak et al. 2006). Inactivation of FOXO1 leads to regulation of neuropeptides that promote negative energy balance (Morton, Gelling et al. 2005, Kim, Pak et al. 2006, Plum, Lin et al. 2009). Our results demonstrated that BM treatment prevents HF diet-induced impairment to downstream leptin signal transduction in the mediobasal and paraventricular nuclei regions of the hypothalamus by maintaining phosphorylation of JAK2, Akt and FOXO1. Furthermore, in the paraventricular nucleus of the hypothalamus, BM prevented HF diet-induced elevations in FOXO1, an important transcription factor responsible for the transcription of neuropeptides that stimulate positive energy balance. This suggests that the paraventricular nuclei of the hypothalamus may have a more important role in the regulation of energy balance via FOXO1.

In addition to leptin-JAK2-Akt-FOXO1 signalling, phosphorylation of JAK2 can also mediate hypothalamic leptin signalling via subsequent phosphorylation of STAT3 (Ladyman and Grattan 2013). However, our results showed no differences between any of the groups in the phosphorylation of STAT3 in the mediobasal and paraventricular nuclei regions of the hypothalamus of mice fed a HF diet for 21 weeks. Despite this, mice fed a HF diet had

significantly higher unphosphorylated STAT3 levels in both the mediobasal and paraventricular regions of the hypothalamus. Unphosphorylated STAT3 has been found to play a role in inflammatory signalling and has been found to accumulate in response to an increase in IL-6 levels in human mammary epithelial (hTERT-HME1) cells (Yang, Liao et al. 2007). Therefore, this suggests that in the mediobasal and paraventricular nuclei regions of the hypothalamus, BM prevents HF diet induced increased levels of unphosphorylated STAT3 resulting in reduced hypothalamic inflammation.

In obesity, downstream hypothalamic leptin signalling is largely impaired due to accentuated activation of the negative regulators, PTP1B and AMPK which inhibit JAK2 activation (Zabolotny, Bence-Hanulec et al. 2002, Bence, Delibegovic et al. 2006, Zhang, Zhang et al. 2008, Su, Jiang et al. 2012). Thus, if there is a dysfunction in this pathway, this regulation of food intake and energy expenditure is disabled. Obese mice induced by a HFD have increased hypothalamic PTP1B levels and leptin resistance (Lam, Covey et al. 2006, White, Whittington et al. 2009, Lu, Wu et al. 2011). In addition, it has been demonstrated that neuronal PTP1B knockout mice have been found to have reduced weight, increased energy expenditure and improved leptin and insulin signalling (Bence, Delibegovic et al. 2006). Activation of hypothalamic AMPK has been demonstrated to increase body weight and food intake in mice (Andersson, Filipsson et al. 2004, Minokoshi, Alquier et al. 2004). Furthermore, pharmacological inhibition of AMPK in the hypothalamus largely enhances leptin signalling (Su, Jiang et al. 2012). In this study, BM prevented HF diet-induced increases in PTP1B and phosphorylation of AMPK proteins in the mediobasal and paraventricular nuclei regions of the hypothalamus. This may have contributed to improved leptin sensitivity through preventing the impairment to downstream hypothalamic leptin-JAK2-Akt-FOXO1 signalling resulting in negative energy balance.

Recently, another important molecule found to be involved in the regulation of food intake and energy expenditure is BDNF. The activation of BDNF in the hypothalamus results in subsequent activation and phosphorylation of TrkB, which also results in the activation and phosphorylation of Akt (Reichardt 2006). An i.c.v injection of BDNF demonstrated reduced appetite and promoted weight loss in mice (Pelleymounter, Cullen et al. 1995). Furthermore, mice with a deletion of the BDNF gene develop severe obesity as a result of overeating (Lyons, Mamounas et al. 1999, Kernie, Liebl et al. 2000). Activation of BDNF and its downstream targets, TrkB and Akt, can be inhibited by PTP1B (Ozek, Kanoski et al. 2014). In addition PTP1B knockout mice have reduced cumulative food intake and body weight following i.c.v BDNF administration (Ozek, Kanoski et al. 2014). Our results demonstrated that BM administration prevented HF diet-induced decreases in BDNF levels in the mediobasal and paraventricular nuclei regions of the hypothalamus. This effect may have been as a result of BM's ability to prevent HF diet induced increases in PTP1B levels in the mediobasal and paraventricular nuclei regions of the hypothalamus, thereby preventing its inhibition of BDNF. However, there were no differences in phosphorylated TrkB levels in both hypothalamic regions between any of the groups. This suggests that energy balance regulation by BDNF may be through its interaction with another substrate in the hypothalamus in promoting its activation of downstream Akt signalling.

Chronic low grade inflammation is a key characteristic of obesity. Activation of a proinflammatory state in rodents fed a HF diet increases the production of the cytokines TNFα and IL-6 in the hypothalamus within the first few days of exposure to this diet (Thaler, Yi et al. 2012). In addition, TNFα administration has been found to increase PTP1B mRNA by 1.4 fold in the hypothalamic arcuate nucleus of diet-induced obese mice (Zabolotny, Kim et al. 2008). In mice fed a HF diet, the activation of phosphorylated JNK is elevated in the mediobasal hypothalamic region of mice fed a HF diet (Benzler, Ganjam et al. 2013). Furthermore, rodents fed a HF diet had

hyperlipidemia, increased food intake, body weight and increased phosphorylation and activation of JNK in the hypothalamus, which was attenuated through inhibition of JNK (De Souza, Araujo et al. 2005). Inhibition of JNK activation has also been found to increase pAkt expression in Lep ob/ob mice (Benzler, Ganjam et al. 2013). We have demonstrated for the first time that BM administration prevents elevations in the activation of the proinflammatory mediator, JNK, and the proinflammatory cytokines TNFα and IL-6 in the mediobasal and paraventricular nuclei regions of the hypothalamus of mice fed a chronic HF diet. This may have contributed to improved leptin sensitivity and hypothalamic leptin signal transduction via inhibition of PTP1B leading to the prevention of HF diet induced increases in energy intake and body weight.

In summary, we have demonstrated that chronic BM administration significantly prevented food intake and body weight gain in mice fed a HF diet. In addition, we found that BM treatment prevented HF diet-induced hyperleptinemia and leptin resistance. Furthermore, our results suggest that BM targets signalling molecules in both the mediobasal and paraventricular nuclei regions of the hypothalamus that promote downstream leptin signalling and prevent inflammation. A proposed model of molecular targets of BM in the hypothalamus in regulating energy balance is summarised in Figure 6. These results therefore identify a novel role for BM as a potential candidate for a future anti-obesity and anti-inflammatory therapeutic. With further research and human clinical trials, the possibility of using BM for the prevention of HF diet-induced development of obesity and associated co-morbidities appears promising.

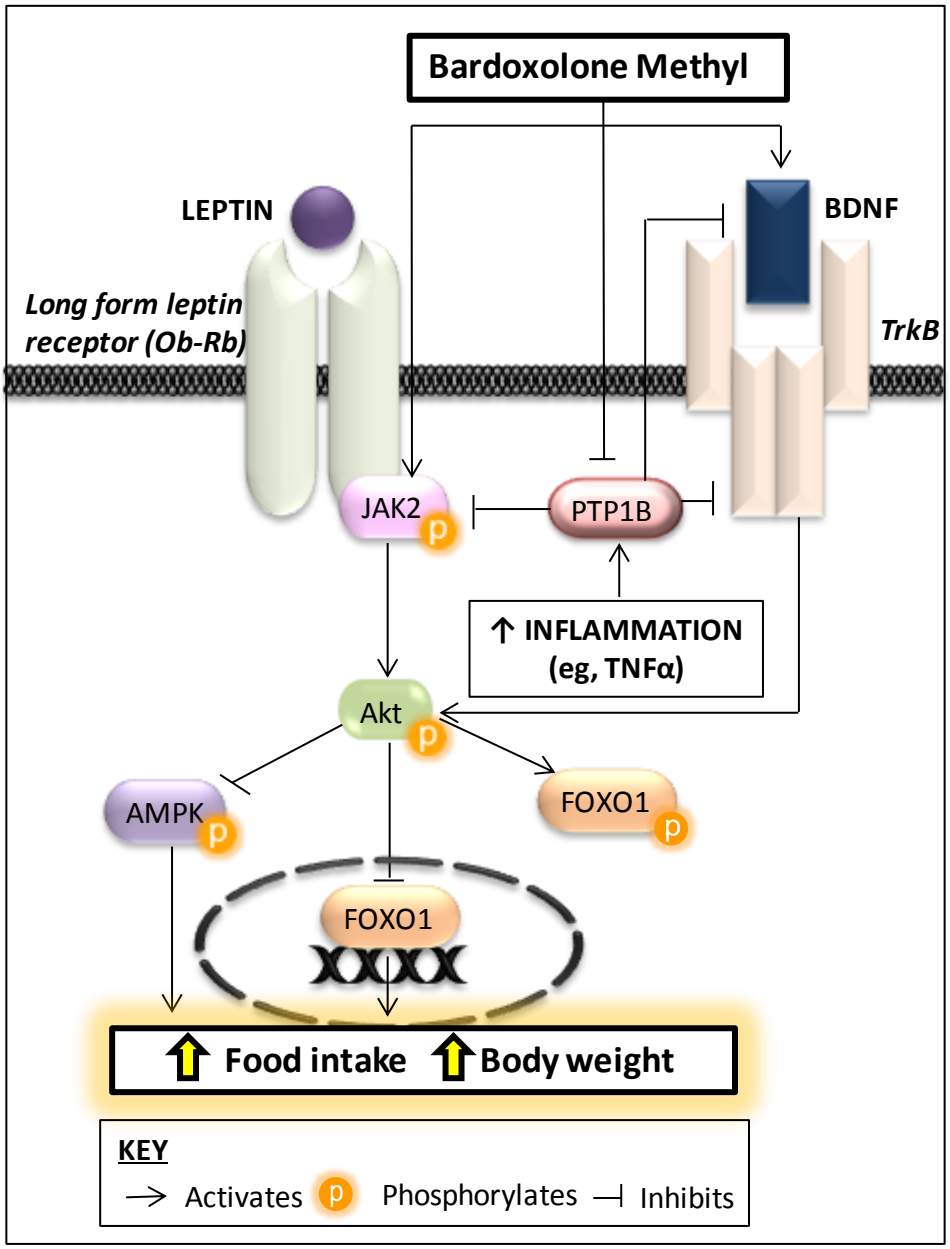

Figure 6: A proposed model of molecular targets of bardoxolone methyl (BM) in the hypothalamus in preventing high-fat (HF) diet-induced obesity, hypothalamic inflammation, and leptin resistance. Our study found that BM prevented HF diet-induced decreases in hypothalamic JAK2-Akt-FOXO1 leptin signalling. Furthermore, our study showed that BM prevented HF diet-induced increases in negative regulators, and inflammatory molecules.

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reviewed and edited the manuscript. Y.Y, A.S, X.H, C.H.L.D and H.W collected data. Y.Y, A.S, and X.H reviewed and edited the manuscript. X.H is the guarantor of this work.

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Chapter Three

Bardoxolone methyl prevents high-fat diet-induced alterations in prefrontal cortex signalling molecules involved in recognition memory

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3.1 Author Contributions

D.Camer was a designer of this study, performed all of the experiments, analysed all the data, and wrote the first draft of the manuscript, which all authors reviewed and approved for publication.

3.2 Collaborator Statement

We hereby declare that the statement in section 3.1 pertaining to the contributions of D.Camer is correct.

Dr Yinghua Yu

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Contents lists available at ScienceDirect

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Bardoxolone methyl prevents high-fat diet-induced alterations in prefrontal cortex signalling molecules involved in recognition memory

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ABSTRACT

High fat (HF) diets are known to induce changes in synaptic plasticity in the forebrain leading to learning and memory impairments. Previous studies of oleanolic acid derivatives have found that these compounds can cross the blood–brain barrier to prevent neuronal cell death. We examined the hypothesis that the oleanolic acid derivative, bardoxolone methyl (BM) would prevent diet-induced cognitive deficits in mice fed a HF diet. C57BL/6J male mice were fed a lab chow (LC) (5% of energy as fat), a HF (40% of energy as fat), or a HF diet supplemented with 10 mg/kg/day BM orally for 21 weeks. Recognition memory was assessed by performing a novel object recognition test on the treated mice. Downstream brain-derived neurotrophic factor (BDNF) signalling molecules were examined in the prefrontal cortex (PFC) and hippocampus of mice via Western blotting and *N*-methyl-D-aspartate (NMDA) receptor binding. BM treatment prevented HF diet-induced impairment in recognition memory ($p < 0.001$). In HF diet fed mice, BM administration attenuated alterations in the NMDA receptor binding density in the PFC ($p < 0.05$), however, no changes were seen in the hippocampus ($p > 0.05$). In the PFC and hippocampus of the HF diet fed mice, BM administration improved downstream BDNF signalling as indicated by increased protein levels of BDNF, phosphorylated tropomyosin related kinase B (pTrkB) and phosphorylated protein kinase B (pAkt), and increased phosphorylated AMP-activated protein kinase (pAMPK) ($p < 0.05$). BM administration also prevented the HF diet-induced increase in the protein levels of inflammatory molecules, phosphorylated c-Jun N-terminal kinase (pJNK) in the PFC, and protein tyrosine phosphatase 1B (PTP1B) in both the PFC and hippocampus. In summary, these findings suggest that BM prevents HF diet-induced impairments in recognition memory by improving downstream BDNF signal transduction, increasing pAMPK, and reducing inflammation in the PFC and hippocampus.

1. Introduction

Obesity is a major risk factor for the development of cognitive decline in neurodegenerative disease such as vascular dementia (Hassing et al., 2002). A number of studies provide direct evidence demonstrating a link between high fat (HF) diet-induced obesity and impairments in learning and memory performance, including a decline in recognition memory (Greenwood and Winocur, 1990, 1996; Heyward et al., 2012). Furthermore, preclinical animal studies have demonstrated that a HF diet reduces synaptic plasticity in the prefrontal cortex (PFC) (Val-Laillet et al., 2011) and hippocampus (Molteni et al., 2002; Wu et al., 2003), which leads to learning and memory impairments (Laroche et al., 2000). A HF diet can further induce cognitive decline by promoting neuroinflammation in the forebrain (Miller and Spencer, 2014). Despite this, therapeutic interventions targeting HF diet-induced cognitive impairment are lacking.

The oleanolic acid synthetic derivative, bardoxolone methyl (BM) has attracted attention due to its potential application in a wide variety of diseases (Camer and Huang, 2014; Camer et al., 2014; Liby and Sporn, 2012; Reisman et al., 2012; Wang et al., 2011). A recent study found that BM can promote dopaminergic neuroprotection via attenuation of the inflammatory mediator, tumour necrosis factor alpha (TNFα), and reactive oxygen species (ROS) production in vitro (Tran et al., 2008). Despite this finding no study has subsequently investigated the effects of BM on the brain in vivo. However, a derivative of BM, CDDO-MA, improved

Abbreviations: HF, high fat; Akt, protein kinase B; PTP1B, protein tyrosine phosphatase 1B; TNFα, tumour necrosis factor alpha; ROS, reactive oxygen species; BM, bardoxolone methyl; PFC, prefrontal cortex; BDNF, brain-derived neurotrophic factor; LTP, long term potentiation; LTM, long term memory; NMDA, *N*-methyl-D-aspartate; AMPK, AMP-activated protein kinase; PTP1B, protein tyrosine phosphatase 1B; HEPES, N-2-hydroxyethylpiperazine-N′-2-ethanesulphonic acid; EDTA, ethylenediaminetetraacetic acid; HSD, honestly significant difference; TrkB, tropomyosin related kinase B; JNK, c-Jun N-terminal kinase.

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0278-5846/

spatial memory and reduced hippocampal amyloid plaques in a mouse model of Alzheimer's disease (Dumont et al., 2009). It has been demonstrated that administration of oleanolic acid has been found to reverse recognition memory impairments in mice (Park et al., 2014). Furthermore, synthetically modifying side chains on oleanolic acid to a derivative form, such as BM, significantly increases its potency (Zhang et al., 2008). Therefore, this suggests that BM has the potential to significantly prevent recognition memory decline, which was examined in our study.

Obesity-induced cognitive impairment is attributed to a reduction of synaptic plasticity (Molteni et al., 2002). Recent evidence has indicated that HF diet-induced impairment in neuronal plasticity may be caused notably by the reduction of brain-derived neurotrophic factor (BDNF) protein expression in the PFC and hippocampus, which are key brain areas in learning and memory (Kanoski et al., 2007). BDNF signalling is a critical pathway for promoting long term potentiation (LTP), a form of synaptic plasticity responsible for long term memory (LTM) formation, and neurogenesis in the forebrain (Noble et al., 2011). Tropomyosin related kinase B (pTrkB) receptor phosphorylation and activation by BDNF leads to a downstream intracellular cascade resulting in activation of protein kinase B (pAkt) signalling (Cunha et al., 2010). Akt signalling regulates the translation and transport of synaptic proteins in order to promote synaptic plasticity in learning and memory (Yoshii and Constantine-Paton, 2007). Along with the activation of TrkB, BDNF also triggers the opening of Na^+ gated ion channels, resulting in an influx of Ca^{2+} and the enhancement of glutamate activation of *N*-methyl-D-aspartate (NMDA) receptors (Rose et al., 2004). The NMDA receptors also play a crucial role in synaptic plasticity with their activation by glutamate leading to the induction of LTP (Bliss and Collingridge, 1993; Cooke and Bliss, 2006). A previous study has reported that a HF diet desensitises NMDA receptors in the hippocampus in mice causing impairment in NMDA-induced long term depression (LTD), suggesting that its alteration may also account for cognitive defects (Valladolid-Acebes et al., 2012). Another important signalling protein that is linked to BDNF is phosphorylated AMP-activated protein kinase (pAMPK). Studies have demonstrated that pAMPK activation increases BDNF expression in the brain (Gomez-Pinilla et al., 2008; Yoon et al., 2008; Zhao et al., 2008), suggesting that its activation plays a crucial role in promoting synaptic plasticity. Furthermore, it has been reported that a HF diet reduces the phosphorylation of AMPK in the hippocampus in rats (Wu et al., 2006). However, the effect of chronically administered BM in preventing HF diet-induced alterations in BDNF signalling, pAMPK, and NMDA receptor neurotransmission in the PFC and hippocampus of mice remains unexplored, and was investigated in this study.

It is widely accepted that consumption of a HF diet and obesity leads to obesity-induced chronic inflammation in a number of tissues, including the brain (Weisberg et al., 2003; Xu et al., 2003). Several rodent studies have demonstrated that chronic inflammation in the brain induced by a HF diet is also associated with a decline in cognitive performance (Morrison et al., 2010; Pistell et al., 2010; Singh et al., 2012). In the forebrain, synaptic plasticity is disrupted by an increased expression of the inflammatory mediators, protein tyrosine phosphatase 1B (PTP1B) (Fuentes et al., 2012) and phosphorylated c-Jun N-terminal kinase (pJNK) (Jiang et al., 2013). However, whether BM administration can prevent HF diet-induced increases in expression of these inflammatory mediators is unknown and therefore was examined in this study.

Although beneficial effects of BM have been demonstrated in animal models and human clinical trials in a variety of tissues (Pergola et al., 2011; Pitha-Rowe et al., 2009), the effect of BM in the central nervous system during HF diet-induced obesity has not been examined previously. Furthermore, no study has yet investigated whether chronic BM treatment can prevent HF diet-induced decline in recognition memory and synaptic plasticity. Therefore, the purpose of the current study was to determine whether chronic oral BM administration in mice fed a HF diet for 21 weeks could prevent impairments to recognition memory. Our findings suggest that chronic BM supplementation may be useful in reducing impairments in recognition memory by improving BDNF downstream signal transduction, increasing phosphorylation of AMPK, and decreasing PTP1B in the PFC and hippocampus. In addition to these effects the BM supplementation in HF diet fed mice prevented alterations in NMDA receptors and the inflammatory mediator pJNK in the PFC, but not the hippocampus. Therefore, the present study suggests that BM prevents HF diet-induced alterations in signalling molecules involved in recognition memory, with a stronger effect on the PFC compared to the hippocampus.

2. Materials and methods

2.1. Animals and HF diet-induced obesity model

Male C57BL/6J mice (12 weeks old) were purchased from the Animal Resource Centre (Perth, Western Australia) and maintained in the animal facility at the University of Wollongong. The experiments were performed in accordance with the *Australian Code of Practice for the Care and Use of Animals for Scientific Purposes*. All procedures were approved by the Animal Ethics Committee, University of Wollongong, Wollongong, Australia (AE 12/15). Mice were housed in environmentally controlled conditions (temperature 22 °C, 12 h light/dark cycle) and 1 week after acclimatisation were randomly divided into 3 groups (n = 7 per group). For the next 21 weeks one group of mice was fed a lab chow (LC) diet (5% of energy as fat; Vella Stock Feeds, Doonside, New South Wales, Australia), and the other two groups a HF diet (40% of energy as fat; SF11-095, Specialty Feeds, Glen Forrest, Western Australia). The fat present in the HF diet consisted of half lard and half sunflower oil. The mice in the treatment group were one of the groups fed a HF diet for 21 weeks, which also received an oral daily dose of BM (10 mg/kg) in their drinking water. We chose the BM dose according to a previous study (Wu et al., 2014). Body weight was measured weekly for the duration of the experiment (final average body weight after 21 weeks: LC, 27.15 g; HF, 40.84 g; HF + BM, 28.13 g). Area under the curve (AUC) for glucose following a glucose tolerance test was measured (AUC glucose: LC, 969.14 mmol/l; HF, 1102.83 mmol/l; HF + BM, 942.75 mmol/l).

2.2. Novel object recognition test

Recognition memory was assessed by performing a novel object recognition test based on a previously described protocol (Fernandez et al., 2012). Briefly, a white open-field square box measuring 55 cm in length, 55 cm in width, and 35 cm in height was used as the experimental apparatus. The open-field box was located in a sound proof room, and lit at approximately 14 lx. The experimental procedure consisted of habituation, training and retention sessions, which were recorded using a video camera placed above the open-field box. All objects and the open-field box were cleaned with 70% ethanol between each mouse. For habituation, mice were individually placed in the box for 10 min to explore the environment in the absence of objects. During the training session, two identical objects (A) were placed at opposing corners of the box, 5 cm from the adjacent wall. Each mouse was then placed in the middle of the open-field box individually and left to explore the objects for 10 min. A mouse was considered to be exploring the object if it was sniffing, touching or facing the object within 2 cm or less, and measurements were recorded in seconds. For the retention session, one familiar object (A) was replaced with one novel object (B) and measurements were taken according to how much time each mouse spent at each object as per the training session. The retention session commenced upon placing the mouse individually in the middle of the open-field box 90 min after its training session, and leaving it to explore for another 10 min. A recognition index was calculated using the formula: Recognition Index = Object B / (Object A + Object B).

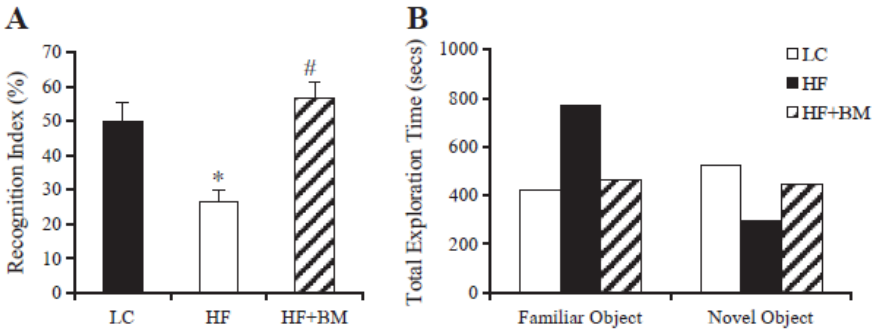

Fig. 1. Effect of chronic bardoxolone methyl (BM) treatment on recognition memory in mice fed a high fat diet for 21 weeks (n = 7 per group). Chronic treatment of BM significantly prevented high fat (HF) diet-induced decline in recognition index in mice (A). Total exploration time between familiar and novel object (B). *, $p = <0.05$ vs. lab chow (LC) group, #, $p = <0.05$ vs. HF group, values are means ± SEM.

2.3. Tissue collection

For tissue analysis (n = 7 per group), mice were euthanised at week 21 of the experiment. Brains were dissected from the mice, snap frozen in liquid nitrogen and stored at −80 °C until use.

2.4. Microdissection

Frozen brain sections containing the PFC and hippocampus regions were cut into 14 μm coronal sections with a cryostat at −18 °C before being mounted on PolylysineTM microscope slides for receptor autoradiography. Further coronal brain sections were cut at 500 μm before the PFC and hippocampus regions were dissected for Western blotting. Sections were collected at levels ranging from Bregma 3.70 mm to −5.20 mm based on a standard mouse brain atlas (Paxinos and Franklin, 2002). The brain sections were stored in −20 °C until use.

2.5. Receptor autoradiography

The procedure for receptor autoradiography to assess NMDA receptor density was based on the protocol described by Newell et al. (2005). Briefly, the brain sections were incubated in 30 nM N-2-hydroxyethylpiperazine-N′-2-ethanesulphonic acid (HEPES) buffer, containing 100 mM glycine and 100 mM glutamate, 1 mM ethylenediaminetetraacetic acid (EDTA) and 20 nM of the radioligand, [^{3}H]MK-801 (specific activity 17.1 Ci/mmol, Perkin Elmer, Boston, Massachusetts, USA) for 2.5 h at room temperature. All solutions for this procedure had a pH of 7.5. Nonspecific binding was determined by incubating adjacent brain sections with [^{3}H]MK-801 in 20 mM MK-801. Following incubation, each section was washed twice for 20 min at 0 °C in a solution of 30 mM HEPES and 1 mM EDTA. The NMDA receptor binding autoradiographic images were taken using a Beta-ImagerTM camera (BioSpace, Paris, France). The sections were scanned at a high-resolution setting for 3.5 h. A series of sections used as standards with a known amount of radioactivity were included in all scans. Quantitative analysis of these images was performed using the β-Image Plus software (version 4, BioSpace).

2.6. Western blot analysis

For protein extraction the frozen PFC and hippocampus tissue samples were homogenised in Nonidet P-40 lysis buffer. The following antibodies were used to quantify specific proteins: BDNF (sc-546), pTrkB (sc-135645), TrkB (sc-377218), pAkt (sc-135650), Akt (sc-1618), pAMPK (sc-33524), AMPK (sc-25792) and pJNK (sc-6254) (Santa Cruz Biotechnology, Dallas, TX); PTP1B (#5311) (Cell Signalling Technology, Beverly, MA). The bands corresponding to the proteins of interest were scanned and the band density analysed using the automatic imaging analysis system, Quantity One (Bio-Rad Laboratories, Hercules, California). All quantitative analyses were normalised to β-actin. Western blots were performed in triplicate for each sample; however, in some cases only two values for each sample were collected. The average of the duplicate/triplicate numbers for each sample was calculated and this number was used for statistical analysis.

2.7. Statistics

Data were analysed using the statistical package SPSS 20 (SPSS, Chicago, IL). Data was first tested for normality before differences between mice fed a LC, HF, and HF supplemented with a BM diet were determined by one-way analysis of variance (ANOVA). This was followed by the post hoc Tukey–Kramer honestly significant difference (HSD) test for multiple comparisons among the groups. A p value of <0.05 was considered statistically significant. Values are expressed as mean ± SEM. Pearson's correlations were used to examine the relationship between recognition index and BDNF levels, recognition index and NMDA receptor density, and AMPK phosphorylation and BDNF levels in the PFC and hippocampus.

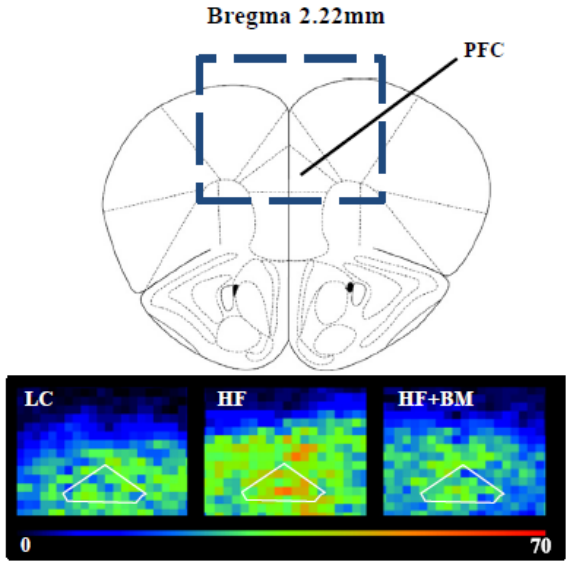

Fig. 2. Effect of chronic bardoxolone methyl (BM) treatment on N-methyl-D-aspartate (NMDA) receptor binding density in the prefrontal cortex (PFC) in mice fed a high fat diet for 21 weeks (n = 7 per group). Chronic treatment of BM significantly prevented high fat (HF) diet-induced alterations in NMDA receptor binding density in the PFC in mice. *, $p = <0.05$ vs. lab chow (LC) group, values are means ± SEM. Scale bar: 0–70 fmol/mg tissue equivalent.

Table 1
NMDAR binding density in mouse forebrain following 21 weeks of LC, HF or HF + BM diet.

Brain area	LC	HF	HF + BM	F value	P value
Prefrontal cortex	14.5 ± 1.7[b]	19.8 ± 0.9[a]	14.3 ± 1.9[b]	2.798	0.038
Hippocampus	16.7 ± 1.9	19.1 ± 2.1	19.5 ± 1.5	0.785	0.473

Values are means ± SEM. LC, lab chow diet, HF, high fat diet, HF + BM, high fat diet and bardoxolone methyl treatment.
[a] $p < 0.05$ vs LC.
[b] $p < 0.05$ vs HF.

3. Results

3.1. Bardoxolone methyl prevented a decline in novel object recognition in mice fed a high fat diet

To assess whether the BM treatment can prevent HF diet-induced long term memory deficits, we performed a novel object recognition test in mice fed a HF diet for 21 weeks. During the training session of the test, the percentage of time spent exploring the identical objects in the open-field was not significantly different among mice fed a LC diet (18.65%), a HF diet (22.83%), and a HF diet treated with BM (17.29%).

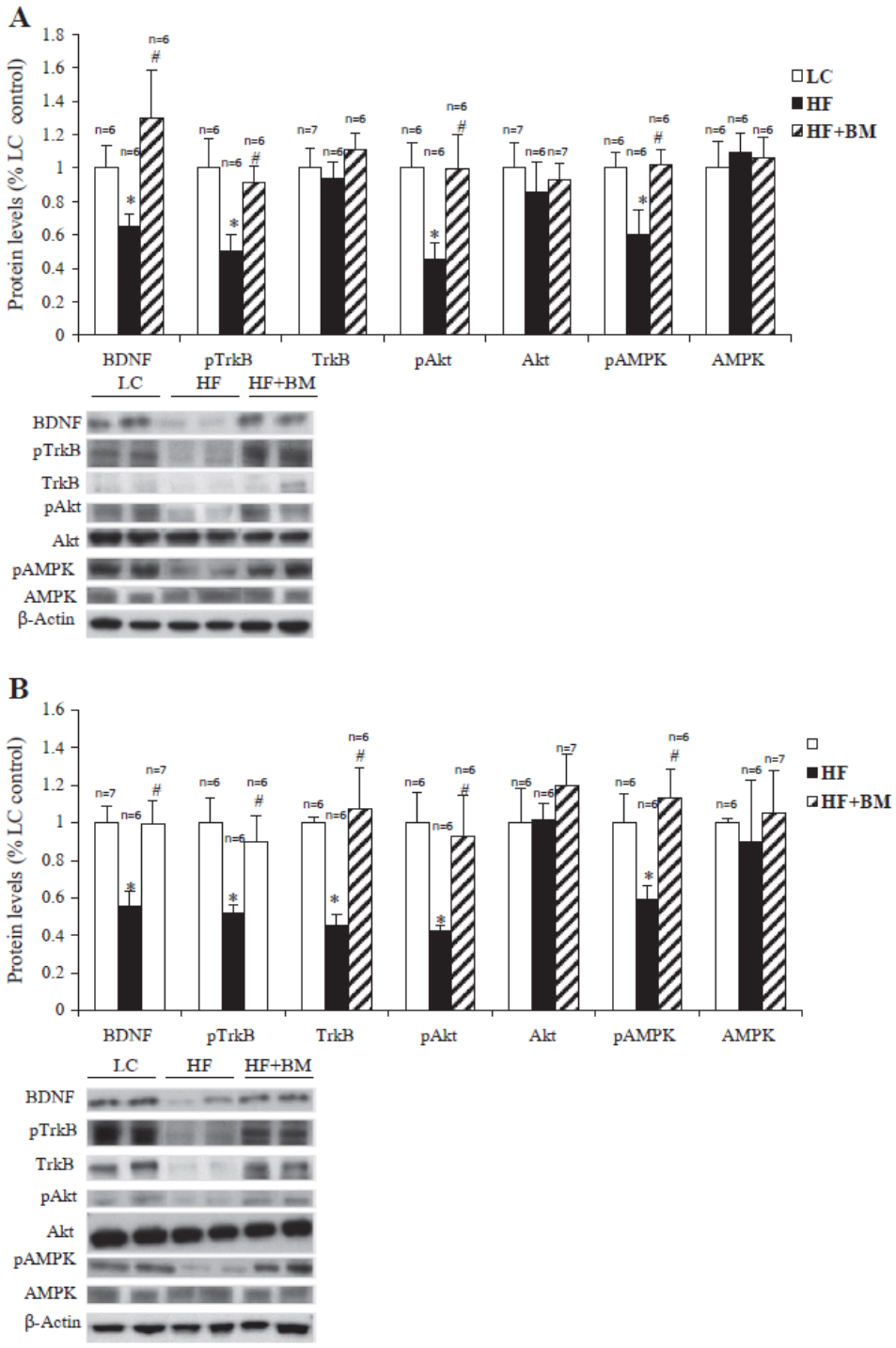

Fig. 3. Effect of chronic administration of bardoxolone methyl (BM) treatment on protein levels of key signalling molecules involved in BDNF signalling of mice (n = 6–7 per group) fed a HF diet for 21 weeks. Chronic treatment of BM significantly prevented a high fat (HF) diet-induced decreases in BDNF, and protein phosphorylation of TrkB, Akt, and AMPK in the prefrontal cortex (PFC) (A) and hippocampus (B). *n* numbers used for quantification are indicated above each column; they are different because of variations in Western blotting between membranes that did not allow us to quantify several lanes for some samples. *, $p = <0.05$ vs. lab chow (LC) group, #, $p = <0.05$ vs. HF group, values are means ± SEM.

However, HF diet fed mice were found to have a significantly reduced recognition index compared to mice fed a LC diet, determined from the novel object recognition test (LC = 50.07%, HF = 26.28%, p = <0.001, Fig. 1). This impairment in memory was prevented through the BM administration, indicated by a significantly higher recognition index (56.82%) in the BM treated mice fed a HF diet compared to the untreated HF diet fed mice (p = <0.001, Fig. 1A). These results show that recognition memory deficits caused by a HF diet may be prevented with BM treatment. In addition, the total sum of exploration time between the familiar and novel object is presented in Fig. 1B (difference in time between familiar and novel object (LC = +19.5%, HF = −62.0%, HF + BM = −4.7%)).

3.2. Bardoxolone methyl prevented an increase in NMDA receptor density in the prefrontal cortex of mice fed a high fat diet

[^{3}H]MK-801 binding density was measured in mouse PFC and hippocampus in order to determine the density of NMDA receptors. NMDA receptor density in the PFC of HF diet fed mice was significantly increased compared with the LC fed mice ([^{3}H]MK-801 receptor binding density difference: 26.81%, p = <0.05, Fig. 2 and Table 1). However, the level of [^{3}H]MK-801 binding to NMDA receptors in BM treated mice fed a HF diet was significantly reduced compared to the HF diet controls in the PFC ([^{3}H]MK-801 binding density difference: 27.76%, p = <0.05, Fig. 2 and Table 1). In mouse hippocampus there were no significant differences in NMDA receptor density among LC, HF and BM groups (Table 1). These results suggest that BM administration in mice fed a HF diet prevents the obesity-induced alteration of NMDA receptor binding density in the PFC, but not in the hippocampus.

3.3. Bardoxolone methyl prevented HF diet-induced decline in downstream BDNF signalling and phosphorylation of AMPK in the forebrain of mice fed a HF diet

We evaluated the effect of BM on the expression of BDNF and its associated signalling molecules in the PFC and hippocampus of HF diet fed mice using Western blotting analysis (n = 6–7 per group for each protein). In both the PFC and hippocampus, Western blot analysis showed that a HF diet reduced BDNF levels and the phosphorylation of TrKB, which was significantly reversed by the BM treatment (p = <0.05, Fig. 3A and B). Furthermore, HF diet-induced decreases in phosphorylation of the proteins Akt and AMPK were prevented by BM administration (p = <0.05, Fig. 3A and B). In addition, BDNF levels were positively correlated to the phosphorylation of AMPK in the PFC (r = 0.674, p = <0.01), and hippocampus (r = 0.798, p = <0.001) (Fig. 4A and B). In examining total protein expression, BM prevented HF diet-induced decreases in TrkB levels in the hippocampus, but not the PFC. There were no significant differences between the total protein expressions of Akt and AMPK in the PFC or hippocampus. These results suggest that BM prevents HF diet-induced decreases in BDNF, phosphorylation of TrkB and the phosphorylation of associated signalling molecules, Akt and AMPK in the PFC and hippocampus.

3.4. Bardoxolone methyl prevented HF diet-induced elevations in the inflammatory mediators, PTP1B and phosphorylation of JNK in mouse prefrontal cortex

The downstream inflammatory mediators, PTP1B and pJNK, were measured in the prefrontal cortex and hippocampus of mice fed a HF diet using Western blotting in order to assess if the BM treatment could prevent neuroinflammation (n = 6–7 per group for each protein). The PTP1B levels were significantly increased in both the PFC and hippocampus, and protein phosphorylation of JNK was significantly increased in the PFC in mice fed a HF diet, which was significantly reduced by BM administration (p = <0.05, Fig. 5A and B). However, no differences were seen in the protein phosphorylation of JNK between any of the groups in the hippocampus (p = >0.05). These results suggest that BM prevents HF diet-induced elevations in inflammatory mediators in the PFC and hippocampus.

3.5. The relationship between recognition index, BDNF levels, and NMDA receptor density in brain regions examined

In the PFC there was a significant positive correlation between recognition index and BDNF levels (r = 0.547, p = <0.05, Fig. 6A and B), and a significant negative correlation between recognition index and NMDA receptor density (r = −0.532, p = <0.05) in the PFC. However, in the hippocampus, there were no significant correlations between recognition index and BDNF levels (r = 0.382, p = 0.118) or between recognition index and NMDA receptor binding density (r = −0.174, p = 0.473) in the hippocampus. This suggests that both NDMA and BDNF expression in the PFC may be related to the changes of recognition memory reported in the different tested groups.

4. Discussion

Rodents fed a HF diet show elevated body weight gain, along with cognitive decline, including impairments in recognition memory (Carey and Gomes, 2014; Heyward et al., 2012; Valladolid-Acebes et al., 2011). In the current study, a chronic HF diet decreased the recognition index in the novel object recognition test, which reflects that recognition memory was impaired in HF diet-induced obesity. Previously, a derivative of BM, CDDO-MA, improved spatial memory and reduced inflammation in the hippocampus in a mouse model of Alzheimer's disease (Dumont et al., 2009). In the present study, we found that BM prevented deficits in recognition memory in mice fed chronic HF diet,

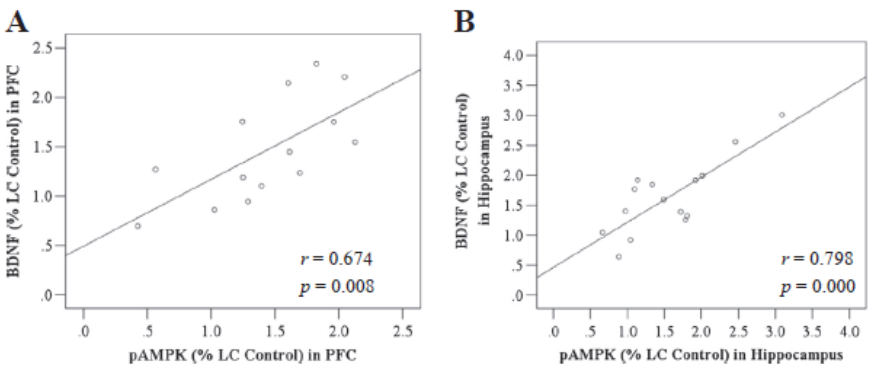

Fig. 4. Correlation between BDNF and AMPK protein phosphorylation in the prefrontal cortex (PFC) (A) and hippocampus (B) in mice fed a high fat (HF) diet and treated with bardoxolone methyl (BM) for 21 weeks (n = 6–7 per group). *, p = <0.05 vs. lab chow (LC) group, values are means ± SEM.

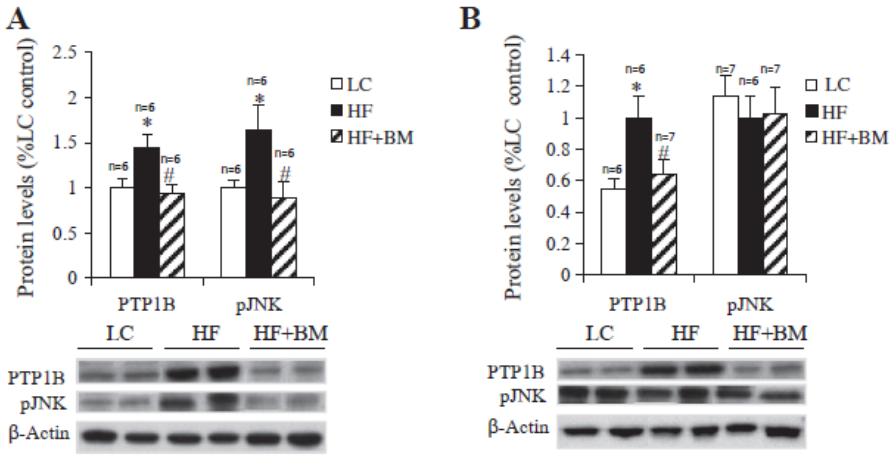

Fig. 5. Effect of chronic administration of bardoxolone methyl (BM) treatment on protein levels of inflammatory mediators of mice (n = 6–7 per group) fed a high fat (HF) diet for 21 weeks. Chronic treatment of BM significantly prevented a HF diet-induced increase in PTP1B and JNK protein phosphorylation in the prefrontal cortex (PFC) (A) and PTP1B in the hippocampus (B). No significant differences were found in the phosphorylation of JNK between any group in the hippocampus. *n* numbers used for quantification are indicated above each column; they are different because of variations in Western blotting between membranes that did not allow us to quantify several lanes for some samples.*, $p = <0.05$ vs. lab chow (LC) group, #, $p = <0.05$ vs. HF group, values are means ± SEM.

suggesting that BM has the potential to improve cognition in obesity and associated neurodegenerative disorders.

In the PFC and hippocampus, signalling through the BDNF pathway promotes neuronal plasticity and neurogenesis, which are both important for learning and memory (Kanoski et al., 2007; Sakata et al., 2013). Rats fed an unrestricted HF diet display decreased BDNF levels in both hippocampus and PFC, which is coupled with impaired discrimination learning (Kanoski et al., 2007). In the brain, BDNF binds to TrkB receptors causing its phosphorylation and subsequent activation of the Akt intracellular signalling cascade to promote synaptic plasticity (Cunha et al., 2010). Our study demonstrated that the BM treatment in mice fed a HF diet for 21 weeks promoted the downstream BDNF signalling cascade by increasing BDNF levels, and protein phosphorylation of TrkB, and Akt in the hippocampus and PFC. This suggests that the actions of BM on the downstream BDNF signalling cascade contributed to improved neuronal plasticity in the hippocampus and PFC of mice fed a HF diet, which further contributed to an improvement in recognition memory. Furthermore, BDNF levels in the PFC were positively correlated to recognition index suggesting that BDNF signalling in the PFC is important for recognition memory.

Several studies have recently suggested an association between BDNF and the phosphorylation of AMPK (Gomez-Pinilla et al., 2008; Yoon et al., 2008; Zhao et al., 2008). Rats who performed a week of exercise demonstrated increases in AMPK protein phosphorylation and BDNF mRNA levels, which was coupled with an enhancement to spatial memory (Gomez-Pinilla et al., 2008). Furthermore, the activation of AMPK has been found to increase the expression of BDNF in the mouse hippocampus (Zhao et al., 2008). Along with increased BDNF levels, our results demonstrated that BM administration increased AMPK protein phosphorylation in the PFC and hippocampus of mice fed a HF diet for 21 weeks. Furthermore, our results found a positive correlation between the phosphorylation of AMPK and BDNF levels in both the PFC and hippocampus. These results suggest that the BM-induced elevation of AMPK protein phosphorylation may modulate BDNF expression, which enhanced synaptic plasticity in the PFC and hippocampus, leading to improved recognition memory.

NMDA receptor activation is important for glutamatergic neurotransmission in learning and memory processes, including recognition memory (Bliss and Collingridge, 1993; Cooke and Bliss, 2006; Warburton et al., 2013). It has been reported that a HF diet induces a desensitisation of NMDA receptors in the brains of mice, resulting in cognitive deficits (Valladolid-Acebes et al., 2012). Therefore, the increased NMDA receptor density we observed in the PFC after a chronic HF diet may reflect a compensation for reduced glutamatergic NMDA receptor function. Importantly, our results found that BM prevented the alteration of NMDA receptor expression in the PFC during a HF diet, which may be involved in its improvement of recognition memory in these mice. Furthermore, the NMDA receptor binding density was negatively correlated to recognition index, suggesting that the HF diet-induced alterations of NMDA receptors in the PFC leads to impairments in recognition memory that can be prevented by BM administration. The PFC and hippocampus are known important brain regions in learning and memory that both utilise the NMDA receptor dependent synaptic plasticity (Banks et al., 2012). Previous studies

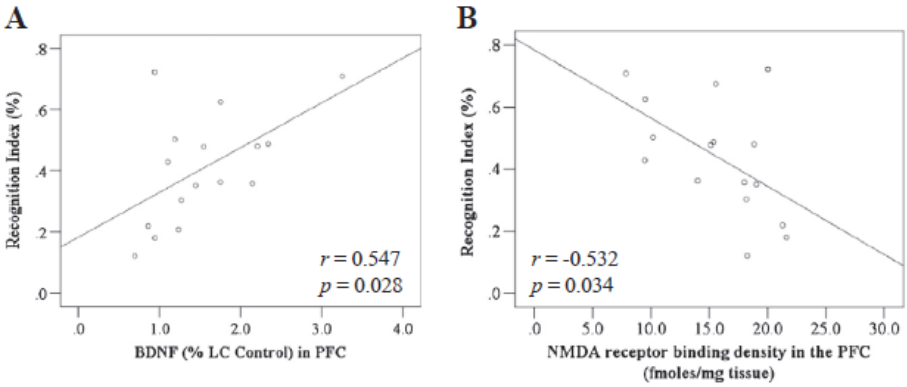

Fig. 6. Correlation between recognition index and BDNF (A), and recognition index and N-methyl-D-aspartate (NMDA) receptor binding density (B) in the prefrontal cortex (PFC) in mice fed a high fat (HF) diet and treated with bardoxolone methyl (BM) for 21 weeks (n = 6–7 per group). *, $p = <0.05$ vs. lab chow (LC) group, values are means ± SEM.

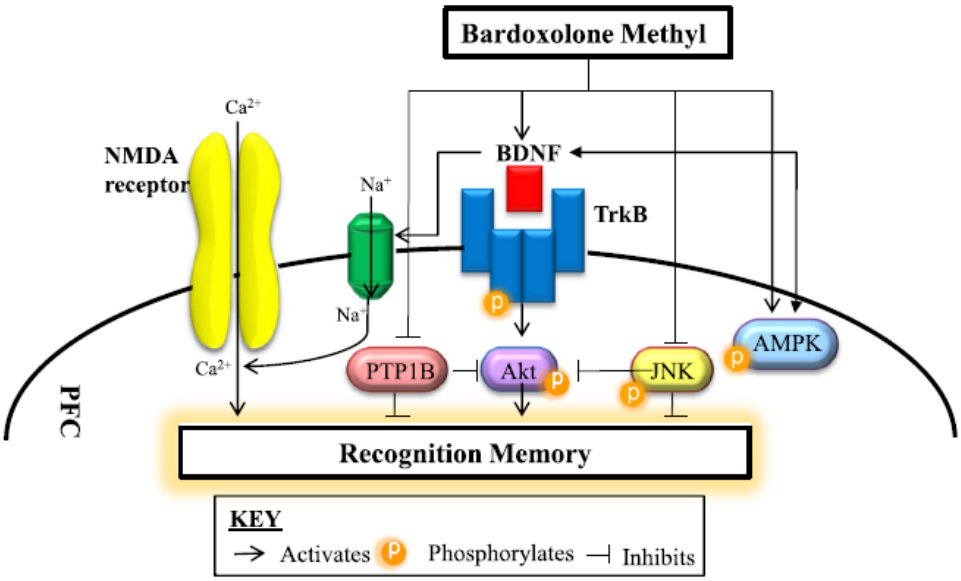

Fig. 7. A proposed model of molecular targets of bardoxolone methyl (BM) in the prefrontal cortex (PFC) in preventing high fat (HF) diet-induced decline in recognition memory. Our study found that BM prevents HF diet-induced decreases in BDNF, protein phosphorylation of TrkB, Akt, and AMPK, and increases in PTP1B and JNK protein phosphorylation in mouse PFC. Furthermore, our study showed that BM prevented HF diet-induced alterations in NMDA receptor density. This suggests that BM can activate BDNF resulting in activation of TrkB receptors, AMPK protein phosphorylation, and NMDA receptor activation. The activation of these targets by BDNF promotes downstream signalling pathways that promote recognition memory.

have demonstrated that the recognition memory can be impaired by lesions on the PFC in monkeys and rats (Bachevalier and Mishkin, 1986; Kolb et al., 1994). On the other hand, studies in rodents showed contradictory results regarding the implication of the hippocampus in recognition memory, reporting that hippocampal lesions may have no effect in object recognition memory in rats (Forwood et al., 2005; Langston and Wood, 2010; Mumby et al., 2002). This suggests that the PFC may have a more influential role in recognition memory compared to the hippocampus in rodents. Our results were in line with these studies, since there were no significant changes in the NMDA receptor binding density in the hippocampus between the groups.

PTP1B and phosphorylation of JNK in the hippocampus and PFC also influence synaptic plasticity, as their activation has been shown to impair learning and memory retention in mice (Fuentes et al., 2012; Wang et al., 2013). Both pJNK and PTP1B are known to cause memory impairments by negatively regulating Akt signalling (Lu et al., 2011; Sunayama et al., 2005). In addition to preventing HF diet-induced decline in Akt protein phosphorylation, our results demonstrated that BM prevented HF diet-induced increases in PTP1B and phosphorylation of JNK in the PFC, and PTP1B in the hippocampus. This suggests that HF diet-induced impairments of learning and memory via these inflammatory mediators were attenuated by BM administration, leading to the promotion of Akt signalling and subsequent improvement of recognition memory.

In conclusion, the findings of this study demonstrate that chronic administration of BM prevents HF diet-induced impairment to recognition memory in mice. Furthermore our results suggest that BM targets signalling molecules in both the PFC and hippocampus that contribute to an improvement in recognition memory. However, it appears that the PFC has a more influential role in this effect. A proposed model of molecular targets of BM in the PFC in promoting recognition memory is summarised in Fig. 7. Our data suggests that a decline in neuronal plasticity in the hippocampus and PFC was attenuated by an increase in BDNF signalling, indicated by increased protein levels of BDNF, and phosphorylation of TrKB and Akt, an increase in AMPK protein phosphorylation, and through a decrease in PTP1B. This was further supported by BM preventing HF diet-induced PFC alterations in the NMDA receptor density and preventing protein phosphorylation of the inflammatory mediator, JNK. Since HF diet-induced obesity has been implicated in the progression of neurodegenerative diseases such as Alzheimer's disease, BM may have beneficial effects in attenuating the progression of cognitive decline. In the future, female mice also need to be studied to rule out gender differences. With further research and eventual human clinical trials, the possibility of using BM for the prevention of HF diet-induced cognitive deficits, including recognition memory impairments, and associated neurodegeneration, appears promising.

Disclosure statement

The authors of this manuscript have nothing to disclose.

Acknowledgements

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Chapter Four

Bardoxolone methyl prevents insulin resistance and the development of hepatic steatosis in mice fed a high-fat diet

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4.1 Author Contributions

D.Camer was a designer of this study, performed all of the experiments, analysed all the data, and wrote the first draft of the manuscript, which all authors reviewed and approved for publication.

4.2 Collaborator Statement

We hereby declare that the statement in section 4.1 pertaining to the contributions of D.Camer is correct.

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Bardoxolone methyl prevents insulin resistance and the development of hepatic steatosis in mice fed a high-fat diet

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ABSTRACT

High-fat (HF) diet-induced obesity is a major risk factor for the development of insulin resistance and hepatic steatosis. We examined the hypothesis that bardoxolone methyl (BM) would prevent the development of insulin resistance and hepatic steatosis in mice fed a HF diet. C57BL/6J male mice were fed a lab chow (LC), HF (40% fat), or HF diet supplemented with 10 mg/kg/day BM orally for 21 weeks. Glucose metabolism was assessed using a glucose tolerance test (GTT) and insulin sensitivity test (IST). Signalling molecules involved in insulin resistance, inflammation, and lipid metabolism were examined in liver tissue via western blotting and RT-PCR. BM prevented HF diet-induced insulin resistance and alterations in the protein levels of protein tyrosine phosphatase 1B (PTP1B), forkhead box protein O1 (FOXO1) and BDNF, and expression of the *insulin receptor (IR)*, *IRS-1* and *glucose-6-phosphatase (G6Pase)* genes. Furthermore, BM prevented fat accumulation in the liver and decreases in the β-oxidation gene, *peroxisomal acyl-coenzyme A oxidase 1 (ACOX)* in mice fed a HF diet. In the livers of HF fed mice, BM administration prevented HF diet-induced macrophage infiltration, inflammation as indicated by reduced IL-6 and signal transducer and activator of transcription 3 (STAT3) protein levels and *TNFα* mRNA expression, and increased *nuclear factor-like 2 (Nrf2)* mRNA expression and nuclear protein levels. These findings suggest that BM prevents HF diet induced insulin resistance and the development of hepatic steatosis in mice fed a chronic HF diet through modulation of molecules involved in insulin signalling, lipid metabolism and inflammation in the liver.

1. Introduction

Obesity is a major risk factor for the development of insulin resistance, type 2 diabetes and hepatic steatosis (Forouhi and Wareham, 2010; Kahn et al., 2006). It is widely accepted that high-fat (HF) diet-induced obesity causes increased fat accumulation, macrophage infiltration and chronic inflammation in peripheral tissues (Weisberg et al., 2003; Xu et al., 2003). Increased fat accumulation and inflammation promote insulin resistance and tissue injury in peripheral tissues involved in glucose and fat metabolism, such as the liver (Weisberg et al., 2003; Xu et al., 2003). A number of studies provide direct evidence demonstrating a link between obesity-associated inflammation and insulin resistance, and hepatic steatosis (Emanuela et al., 2012; Ginsberg, 2006; Qureshi and Abrams, 2007). However, there is a need to develop novel therapeutic approaches targeting hepatic inflammation and to improve obesity-induced insulin resistance and hepatic steatosis.

The activation of inflammatory molecules can promote the expression of the negative regulators of insulin signalling, protein tyrosine phosphatase B (PTP1B) and SOCS3 (Hong et al., 2001; Zabolotny et al., 2008). PTP1B levels are increased in the liver of HF diet-induced obese mice, which contributes to the development of insulin resistance by reducing insulin signalling through inhibition of insulin receptor (IR) and insulin receptor substrate 1 (IRS-1) activation (Goldstein et al., 2000; Lam et al., 2006). SOCS3 is another important molecule which impairs insulin signal transduction in the liver through its inhibition of the binding of IR to IRS-1 (Ueki et al., 2004). Furthermore, activation of hepatic insulin signalling results in the inactivation of forkhead box protein O1 (FOXO1), which

Abbreviations: ACOX, peroxisomal acyl-coenzyme A oxidase 1; BDNF, Brain-derived neurotrophic factor; BM, bardoxolone methyl; CDDO-Im, 1-[2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oyl]imidazole; FAS, fatty acid synthase; FOXO1, Forkhead box protein O1; HF, high fat; LC, lab chow; GTT, glucose tolerance test; G6Pase, glucose-6-phosphatase; IKKβ, Inhibitor of nuclear factor kappa-B kinase subunit beta; IKKε, Inhibitor of nuclear factor kappa-B kinase subunit epsilon; IR, insulin receptor; IST, insulin sensitivity test; NFκB, Nuclear factor kappa-B; Nrf2, Nuclear factor-like 2; PTP1B, Protein tyrosine phosphatase 1B; SCD1, stearoyl-CoA desaturase 1; STAT3, Signal transducer and activator of transcription 3.

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is a transcription factor inhibiting genes such as *glucose-6-phosphatase (G6Pase)* for endogenous glucose production via gluconeogenesis (German et al., 2009; Nakae et al., 2001). When insulin signalling is impaired, through inhibition by PTP1B or SOCS3, and activation of FOXO1, this leads to the promotion of glucose production and a reduction in glucose reuptake, leading to glucose intolerance and insulin resistance in obesity (German et al., 2009; Nakae et al., 2001). Brain-derived neurotrophic factor (BDNF) also plays an import role in insulin action as it has been found to modulate hepatic glucose metabolism via its actions on glucokinase (GK) in obese insulin resistant rats (Kuroda et al., 2003). In the liver, GK enhances glycolysis, resulting in reduced blood glucose levels (Hariharan et al., 1997).

A HF diet is known to cause fat accumulation in the liver, which can progressively worsen to hepatic steatosis (Marchesini et al., 2001). Hepatic lipid homeostasis is regulated by a number of genes that promote lipogenesis, including *ACC*, *FAS* and *SCD1*, and β oxidation, such as *ACOX* (Musso et al., 2009). Hepatic fat accumulation leads to macrophage infiltration which promotes the production of pro-inflammatory cytokines, such as interleukin-6 (IL-6), tumour necrosis factor alpha (TNFα) and IL-1 β (McArdle et al., 2013). Increased IL-6 has been found to enhance inflammatory signalling by increasing signal transducer and activator of transcription 3 (STAT3) levels, which promotes cytokine dependent signalling by increasing the expression of inflammatory genes such as IL-6 (Yang et al., 2007). In addition, inhibitor of nuclear factor kappa-B kinase subunit beta (IKKβ) and inhibitor of nuclear factor kappa-B kinase subunit epsilon (IKKε) are important pro-inflammatory signalling molecules upstream of the transcription factor, nuclear factor kappa-B (NFκB), which promote PTP1B and SOCS3 activation (Hong et al., 2001; Napetschnig and Wu, 2013; Zabolotny et al., 2008).

The oleanolic acid synthetic derivative, bardoxolone methyl (BM), has attracted wide attention due to its anti-inflammatory effects (Liby and Sporn, 2012; Reisman et al., 2012; Wang et al., 2011). Its ability to directly up-regulate the potent anti-inflammatory molecule, nuclear factor-like 2 (Nrf2), has demonstrated therapeutic benefits in human clinical trials for treating chronic kidney disease and advanced solid tumours (Hong et al., 2012; Liby et al., 2007; NIH, 2012; Pergola et al., 2011). BM has also been found to directly influence the activity of pro-inflammatory signalling through IKKβ (Ahmad et al., 2006). Furthermore, a recent study demonstrated that 2 week administration of BM decreased hepatic inflammation in diet-induced obese mice (Saha et al., 2010). In addition, previous studies have demonstrated that oral administration of a derivative of BM, 1-[2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oyl]imidazole (CDDO-Im), prevents HF diet-induced obesity and attenuates diabetes in mice (Shin et al., 2009; Uruno et al., 2013). In this study, we investigated whether chronic oral BM administration in mice fed a HF diet for 21 weeks could prevent insulin resistance and liver injury in mice fed a HF diet. We also examined signalling molecules involved in insulin resistance, inflammation, and lipid metabolism in liver tissue.

2. Materials and methods

2.1. Animals and HF diet-induced obesity model

Male C57BL/6J mice (12 weeks old) were purchased from the Animal Resource Centre (Perth, Western Australia) and maintained in the animal facility at the University of Wollongong. The experiments were performed in accordance with the *Australian Code of Practice for the Care and Use of Animals for Scientific Purposes*. All procedures were approved by the Animal Ethics Committee, University of Wollongong, Wollongong, Australia (AE 12/15). Mice were housed in environmentally controlled conditions (temperature 22 °C, 12 hr light/dark cycle) and 1 week after acclimatisation were randomly divided into 3 groups (n = 7 per group). For the next 21 weeks one group of mice were fed a lab chow (LC) diet (5% of energy as fat; Vella Stock Feeds, Doonside, New South Wales, Australia), and the other two groups a HF diet (40% of energy as fat; SF11-095, Specialty Feeds, Glen Forrest, Western Australia), and mice in the treatment group were fed a HF diet for 21 weeks and an oral daily dose of BM (10 mg/kg) in their drinking water (Table 1) (final average body weight after 21 weeks: LC, 27.15 g; HF, 40.84 g; BM, 28.13 g). HOMA-IR was calculated using the formula, (Fasting Insulin × Fasting Glucose)/22.5.

Table 1
Composition of the high fat and lab chow diets.

	High fat diet	Lab chow diet
Total energy (kcal/100 g)		
Fat	40	5
Carbohydrate	45	75
Protein	15	20

Typical ingredients.
High fat diet: Casein (acid), sucrose, lard, sunflower oil, cellulose, wheat starch, dextrinized starch, minerals, and vitamins.
Lab chow diet: Cereal grains, meat offal meal, fish offal meal, whey powder, vegetable oils, soybean protein, cereal offal, corn offal, minerals, and vitamins.

2.2. Glucose tolerance test

Mice were fasted overnight (16 hrs) before a glucose tolerance test (GTT) was performed to assess glucose clearance, following an intraperitoneal (i.p.) injection of glucose (0.5 g/kg; Sigma-Aldrich, St. Louis, MO). Blood samples were taken from the tail vein before and 30, 60 and 120 minutes following the injection of glucose. Blood glucose was measured using an Accu-Chek glucometer (Roche Diagnostics GmbH Mannheim, Germany).

2.3. Insulin sensitivity test

Mice were fasted for 5 hours before an insulin sensitivity test (IST) was performed to assess glucose clearance, following an i.p. injection of insulin (0.75 U/kg; Sigma-Aldrich, St. Louis, MO). Blood samples were taken from the tail vein before and at 30, 60 and 120 minutes following the injection of insulin. Blood glucose was measured using an Accu-Chek glucometer (Roche Diagnostics GmbH Mannheim, Germany).

2.4. Tissue collection and sample preparations

For tissue analysis, mice were euthanized at week 21 of the experiment. Tissue was dissected from the mice and immediately frozen in liquid nitrogen before being stored at –80 °C.

2.5. Oil Red O staining

Oil Red O staining was used to examine hepatic lipid accumulation as described previously (Kudo et al., 2007). Briefly, frozen liver sections (10 µm) were stained with 0.5% Oil Red O (Sigma-Aldrich) for 15 minutes and then washed. Three fields from three sections of each mouse were viewed under a Leica microscope, and digital photographs were captured. Image J software (http://imagej.nih.gov/ij/download.html) was used to quantify the staining, which corresponds to the percentage of stained lipid droplets on an area of each slide (Mehlem et al., 2013).

2.6. Haematoxylin and eosin (H&E) staining

To determine the degree of liver damage fresh frozen liver sections (10 µm) were stained with haematoxylin and eosin for 30 s each. Three fields from three sections of each mouse were viewed

under a Leica microscope and digital photographs were captured. The histological parameters of steatosis and ballooning were scored according to the method described by Kleiner et al. (2005). The steatosis grades were as follows: 0, <5%; 1, 5%–33%; 2, >33%–66%; 3, >66%. The ballooning classifications were grouped as: 0, no ballooning cells; 1, few ballooning cells; 2, many cells/prominent ballooning.

2.7. Immunohistochemistry

Liver sections fixed in 10% formalin were embedded in paraffin before being sectioned (5 µm) onto polylysine slides. Slides were incubated overnight at 4 °C with anti-rabbit F4/80 (1:150 Santa Cruz Biotechnology, Dallas, TX) diluted in blocking buffer as described previously (Dinh et al., 2015). Three fields from three sections of each mouse were viewed under a Leica microscope and digital photographs were captured. Image J software was used to quantify the area of F4/80 immunoreactivity on each slide.

2.8. Extraction of nuclear and cytosolic proteins

Nuclear and cytosolic proteins were extracted from liver tissue as described by Mobasher et al. (2013). Briefly, liver tissue was homogenized in a solution containing 10 mM HEPES–KOH (pH 7.9), 10 mM KCL, 1.5 mM $MgCl_2$, 0.5 mM DTT, 0.2 mM PMSF, and protease and phosphatase inhibitors (buffer A) before incubation on ice, vortexing and centrifugation. Following centrifugation, the supernatant containing the cytosolic fraction was collected and frozen at –80 °C until use. The remaining pellet was resuspended in a solution containing 20 mM HEPES–KOH (pH 7.9), 400 mM NaCl, 1.5 mM $MgCl_2$ 0.2 mM EDTA, 15% glycerol, 0.5 mM DTT, 0.2 mM PMSF and protease and phosphatase inhibitors (buffer B) before further centrifugation. Following multiple washes with buffer B and centrifugation of the pellet, the supernatant containing the nuclear fraction was collected and stored at –80 °C until use.

2.9. Western blot analysis

For total protein extraction, the frozen liver tissue was homogenized in Nonidet P-40 lysis buffer. The following antibodies were used for western blotting: Nrf2 (sc-722), IL-1β (sc-7884), IL-6 (sc-7920) and BDNF (sc-546) (Santa Cruz Biotechnology, Dallas, TX); pIKK (#2697), STAT3 (#4904), FOXO1 (#2880), SOCS3 (#2932), and PTP1B (#5311) (Cell Signalling Technology, Beverly, MA). Both nuclear and cytosolic protein levels of Nrf2 were analyzed. The bands corresponding to the proteins of interest were scanned and the band density was analyzed using the automatic imaging analysis system, Quantity One (Bio-Rad Laboratories, Hercules, California) as described in our previous study (Camer et al., 2015). All quantitative analyses for total and cytosolic proteins were normalized to β-actin. Nuclear proteins were normalized to Lamin B.

2.10. Luminex assay

Blood was collected in EDTA tubes from mice following euthanasia. Following centrifugation, plasma was extracted, collected and stored at –80 °C. Plasma insulin levels were measured using Luminex assay kits according to the manufacturer's instructions (BioRad Diabetes Kit, Sydney).

2.11. RNA isolation and RT-PCR

Total RNA was extracted from mouse liver using the Aurum total RNA mini kit (Bio-Rad Laboratories, Hercules, CA) before being reversed transcribed to complementary first strand DNA with a high-capacity cDNA reverse transcription kit (AB Applied Biosystems, California, USA) according to the manufacturer's directions. Quantitative real-time PCR (RT-PCR) was performed using a Lightcycler 480 real time PCR system (F.Hoffmann-La Roche Ltd, Switzerland). A 20 µl final reaction volume containing cDNA sample and SYBR green I master mix was used to perform the experiment. Briefly, amplification was carried out with 45 cycles of 95 °C for 10 s, 60 °C for 30 s and 72 °C for 30 s. The expression of mRNA was normalized to an internal control, GADPH. The degree of mRNA expression was calculated using the comparative threshold cycle value (Ct) method, using the formula $2^{-\Delta\Delta Ct}$ (where ΔΔCt =ΔCt sample – ΔCt reference) as described previously (Livak and Schmittgen, 2001). The primers used are listed in Supplementary Table S1.

2.12. Statistics

Data were analyzed using the statistical package SPSS 20 (SPSS, Chicago, IL). Data were first tested for normality using a Kolmogorov–Smirnov normality test. Differences between mice fed a LC, HF, and HF plus BM diet were then determined by one-way analysis of variance (ANOVA). This was followed by the post hoc Tukey–Kramer honestly significant difference (HSD) test for multiple comparisons among the groups. A p value of <0.05 was considered statistically significant. Values are expressed as the mean ± SEM.

3. Results

3.1. Bardoxolone methyl treatment prevented HF diet-induced insulin resistance

To explore the role of BM in glucose homeostasis and insulin sensitivity, glucose tolerance tests (GTTs) and insulin sensitivity tests (ISTs) were performed (Fig. 1A and C). HF diet fed mice had significantly higher blood glucose levels during fasting (0 minute) and 120 minutes following an i.p. injection of glucose compared to LC fed mice. However, administration of BM normalized blood glucose levels at 120 minutes in the GTT test in HF diet fed mice (–18.07%, $p = 0.015$) with significance confirmed with area under the curve (AUC) analysis (Fig. 1B). However, BM did not prevent HF diet-induced increases in fasting blood glucose levels ($p > 0.05$). Consistent with the effect of BM on improving glucose clearance, BM treatment also reduced blood glucose levels during the IST in mice fed a HF diet (Fig. 1C). HF diet-fed mice had significantly higher blood glucose levels at fasting and 30, 60 and 120 minutes following insulin injection compared to LC fed mice. BM treatment significantly decreased blood glucose levels at 30 and 60 minutes post i.p. insulin injection (Fig. 1C) in the mice fed a HF diet, with significance confirmed with area under the curve (AUC) analysis (Fig. 1D) (blood glucose levels 30 minutes following i.p. insulin injection: –34.23%, $p = <0.001$; blood glucose levels 60 minutes following i.p. insulin injection: –25.92%, $p = 0.048$).

Fasting plasma insulin levels were examined to determine if BM could prevent HF diet-induced hyperinsulinemia. As expected, mice fed a HF diet for 21 weeks had significantly elevated plasma insulin levels compared to LC fed mice, which was attenuated by BM administration (Fig. 1E). To determine if BM treatment could prevent HF diet-induced insulin resistance, HOMA-IR was calculated. HF diet-fed mice were found to have a significantly elevated HOMA-IR compared to LC group (Fig. 1F). However, BM administration in HF diet fed mice significantly prevented this increase in HOMA-IR. These results suggest that BM can prevent hyperinsulinemia and insulin resistance induced by a chronic HF diet.

We evaluated the effect of BM on the expression of molecules involved in insulin resistance and glucose metabolism in the liver using western blotting and RT-PCR analysis. Western blot showed that a HF diet elevated hepatic PTP1B and FOXO1, and reduced BDNF

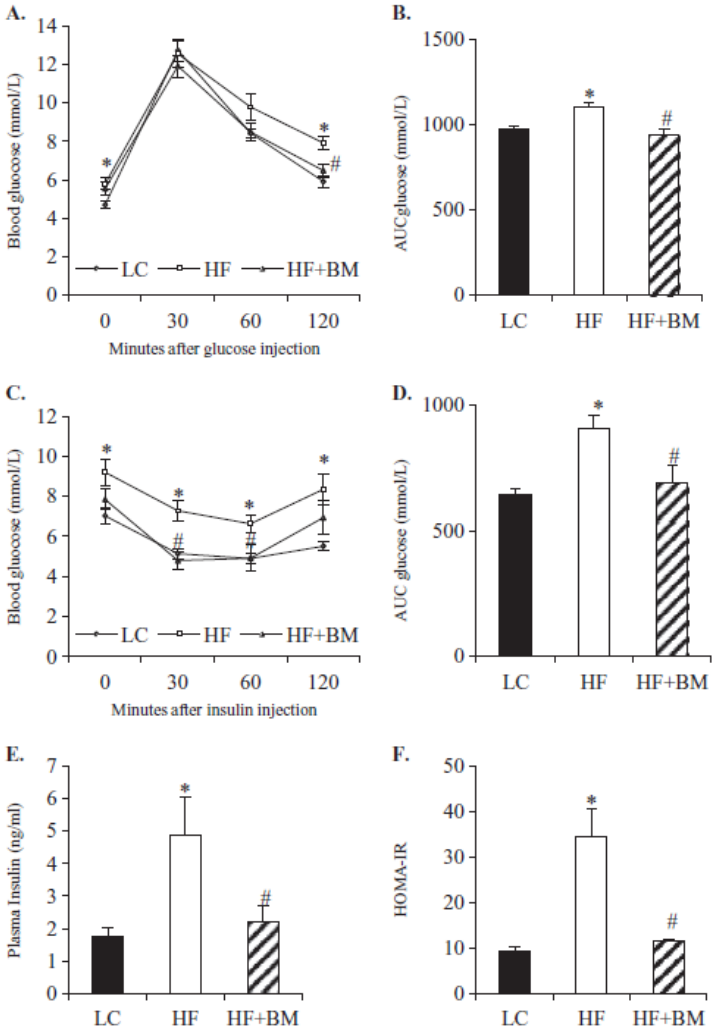

Fig. 1. Effect of chronic administration of bardoxolone methyl (BM) on glucose tolerance (A, B), insulin sensitivity (C, D), hyperinsulinemia (E) and HOMA-IR (F) in mice fed a high fat (HF) diet for 21 weeks (n = 7 per group). *, $p = <0.05$ vs. lab chow (LC) group, #, $p = <0.05$ vs. HF group values are means ± SEM.

protein expression, which was significantly reversed by BM treatment (Fig. 2A). No significant differences in protein expression of SOCS3 were found between any groups (Fig. 2A). RT-PCR analysis found that a HF diet significantly reduced *IR* and *IRS-1* mRNA expression and significantly increased *G6Pase* and *GK* mRNA expression (Fig. 2B). BM treatment significantly prevented HF diet-induced decreases in *IR* and *IRS-1* and increases in *G6Pase* mRNA expression (Fig. 2B). However, BM was unable to prevent HF diet-induced decreases in the mRNA expression of *GK* (Fig. 2B). These data suggest that BM prevents the development of HF diet induced hepatic insulin resistance by regulating the insulin signalling proteins, FOXO1, PTP1B and BDNF, and *IR*, *IRS-1* and *G6Pase* genes to promote insulin signalling, and reduce glucose production.

3.2. Bardoxolone methyl prevented HF diet-induced hepatic fat accumulation and alterations in fatty acid metabolism-related genes

Hepatic steatosis is a severe fatty liver disease caused by the accumulation of fat deposits in hepatocytes in liver tissue (Marchesini et al., 2001). On a histological level, the diagnostic criteria for hepatic steatosis include the presence of steatosis and ballooning (Neuschwander-Tetri and Caldwell, 2003). Upon gross examination, we found that the BM treated livers weighed less and were visibly less steatotic than the livers from the HF diet-fed group (final liver weight –23.18%, $p = <0.001$, Fig. 3A and B). We performed haematoxylin and eosin (H&E) and oil red O staining to examine the effects of BM on hepatic lipid content, ballooning and steatosis (Fig. 3C). Histological examination revealed that the hepatocytes of HF diet-fed mice were enlarged and contained large cytoplasmic lipid droplets compared to LC fed mice (lipid content area (%) difference: –61.94%, $p = <0.001$; ballooning difference: –72.78%, $p = <0.001$; steatosis difference: –58.65%, $p = <0.001$). This change in hepatic cellular morphology was prevented by BM treatment, where the percentages of hepatic lipid area, ballooning, and steatosis were significantly lower compared to the HF diet fed group (lipid content area (%) difference: –61.94%, $p = <0.001$; ballooning difference: –72.78%, $p = <0.001$; steatosis difference: 62.02%, $p = <0.001$) (Table 2).

Fatty acid metabolism-related genes in the liver were measured using RT-PCR in order to assess if these markers were responsible for BM's ability to prevent HF diet-induced hepatic fat accumulation (Fig. 3D). The results showed that BM prevented HF diet-induced decreases in the β oxidation gene, *ACOX* (HF vs. LC difference: –98.84%, $p = <0.001$; HF vs. BM difference: –94.02%, $p = <0.001$). However the expression of *ACOX* was still significantly higher in the LC group compared to HF diet-fed mice treated with BM (LC vs. BM difference: –80.61%, $p = <0.001$). Furthermore, the levels of the lipogenic genes *SCD1* and *FAS* were significantly lower in the BM group compared to the untreated HF diet group. However,

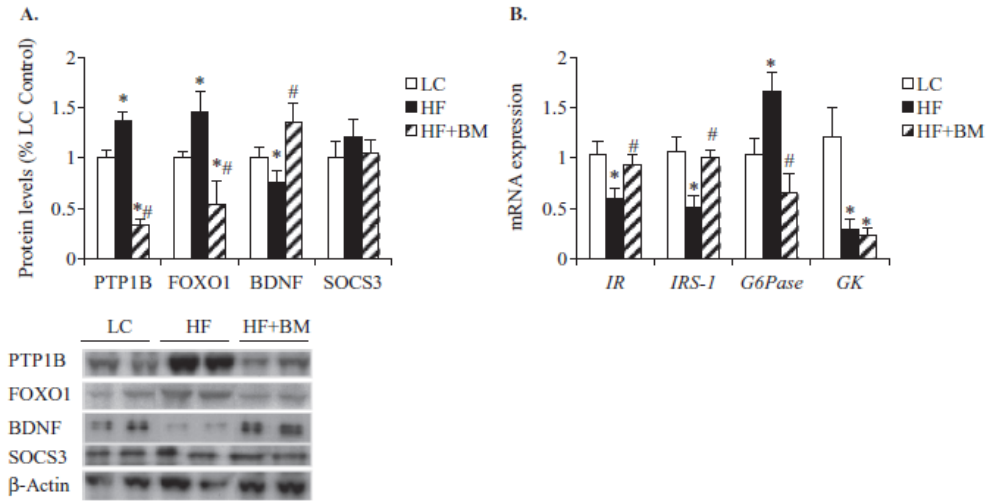

Fig. 2. Effect of chronic bardoxolone methyl (BM) treatment on the hepatic insulin signalling proteins (A) FOXO1, PTP1B, BDNF and SOCS3, and genes (B) *IR*, *IRS-1*, *G6Pase* and *GK*, in the livers of mice fed a high fat (HF) diet for 21 weeks (n = 7 per group). *, $p = <0.05$ vs. lab chow (LC), #, $p = <0.05$ vs. HF group, values are means ± SEM.

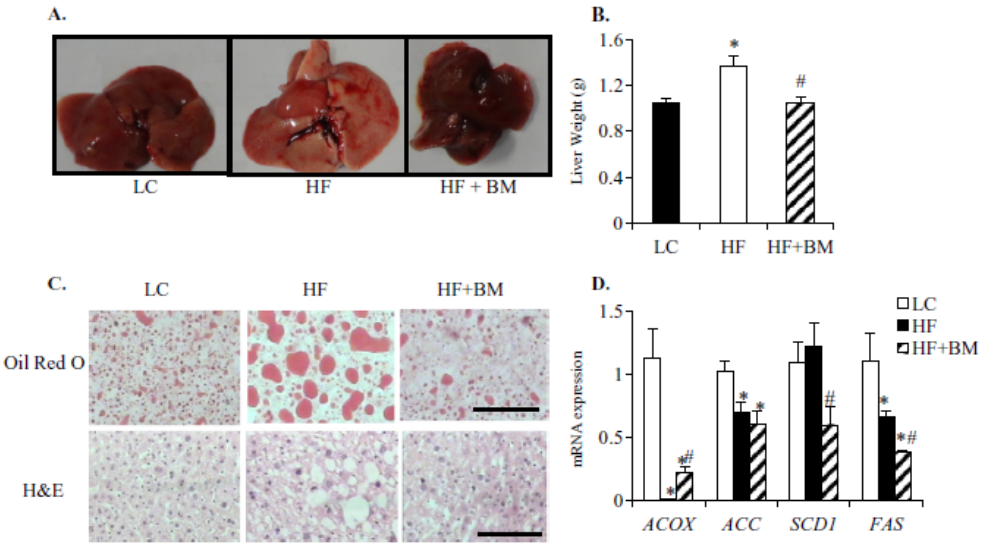

Fig. 3. Effect of chronic administration of bardoxolone methyl (BM) on liver appearance (A), weight (B), lipid content (C), and expression of fatty acid metabolism-related genes (D) *FAS*, *SCD1*, *ACC* and *ACOX*, in the livers of mice (n = 7 per group) fed a high fat (HF) diet for 21 weeks. *, $p = <0.05$ vs. lab chow (LC) group, #, $p = <0.05$ vs. HF group, values are means ± SEM. Scale bar = 50 μm.

Table 2
Liver histopathology in mice following 21 weeks of LC, HF or HF + BM diet.

Parameter	LC	HF	HF + BM	F value	P value
Lipid content area (%)	12.46 ± 1.92[b]	32.74 ± 3.32[a]	12.46 ± 1.88[b]	23.144	<0.001
Ballooning	0.43 ± 0.17[b]	1.58 ± 0.15[a]	0.43 ± 0.14[b]	17.6	<0.001
Steatosis	0.86 ± 0.23[b]	2.08 ± 0.19[a]	0.79 ± 0.21[b]	10.793	<0.001

Lipid content area (%): The percentage of lipid present on a viewed field on a microscope. Hepatocyte ballooning: 0, none; 1, few ballooning cells; 2, many cells/prominent ballooning. Liver steatosis: 0: <5%; 1: 5%–33%; 2: >33%–66%; 3: >66%.Values are means ± SEM.
LC, lab chow diet, HF, high fat diet, HF+BM, high fat diet and bardoxolone methyl treatment.
[a] $p < 0.05$ vs. LC.
[b] $p < 0.05$ vs. HF.

FAS expression in the LC group was significantly higher than both the HF diet group and BM group, and there were no significant differences between the LC and HF diet groups in *SCD1* mRNA expression. In addition, there were no significant differences between *ACC* mRNA expression in the HF and BM groups. However, *ACC* mRNA expression was significantly lower in BM treated mice compared to LC fed mice. These results suggest that BM prevents HF diet-induced fat accumulation in the liver by increasing β oxidation and inhibiting genes involved in lipogenesis.

3.3. *Bardoxolone methyl prevented HF diet-induced hepatic macrophage infiltration and inflammation*

Along with fat accumulation, hepatic steatosis is characterized by a pro-inflammatory state in liver tissue (Marchesini et al., 2001). To investigate the effect of BM on macrophage accumulation in HF diet fed mouse livers, we performed immunohistochemistry with anti-F4/80 antibody (Fig. 4A). We found that macrophage numbers increased in the livers of HF diet-fed mice as indicated by accumulation of F4/80 positive cells. BM administration significantly prevented an increase in the number of F4/80 positive cells in the livers of HF diet-fed mice (Fig. 4B). The hepatic levels of the pro-inflammatory cytokine, IL-6, and signalling molecule, STAT3, were significantly increased in HF diet-fed mice. However, this HF diet-induced increase in IL-6 and STAT3 was prevented by BM treatment (Fig. 4C). There were no significant differences in the expression of hepatic pIKK or IL-1β between the groups. In addition, nuclear protein levels of Nrf2 were significantly reduced in the livers of HF diet-fed mice. However, this reduction was significantly prevented by BM treatment (Fig. 4D). There were no significant differences in hepatic cytosolic Nrf2 protein levels among any of the groups. Furthermore, RT-PCR analysis showed a significant increase in *TNFα* and *IL-6* mRNA expression, and decrease in *Nrf2* mRNA expression in mice fed a HF diet (Fig. 4E). The alterations in *TNFα* and *Nrf2* mRNA levels were significantly prevented by BM administration. However, BM treatment was unable to prevent HF diet-induced elevations in *IL-6* mRNA expression. No significant differences were found in the mRNA expression of *IKKβ* and *IKKε* between any of the groups. These results suggest that BM prevents the development of HF diet induced hepatic macrophage infiltration by regulating pro-inflammatory signalling molecules and activating Nrf2 in the liver.

4. Discussion

Rodents fed a HF diet show fat accumulation, low grade inflammation and insulin insensitivity in peripheral tissues, including the liver (Weisberg et al., 2003; Xu et al., 2003). BM has recently received considerable attention because of its anti-inflammatory, antioxidant, and blood glucose lowering effects (Saha et al., 2010). Although a recent study has investigated the acute anti-inflammatory effects of BM in diet-induced obese mice (Saha et al., 2010), the actions of BM on insulin signalling, fat accumulation and inflammation in the livers of mice fed a chronic HF diet have not been examined previously. In the current study, we found that BM not only prevents HF diet-induced hepatic insulin resistance and inflammation, but it also reduces liver injury by preventing the development of fat accumulation and progression to hepatic steatosis.

A number of studies suggest that obesity-induced inflammation plays an important role in the development of insulin resistance (Boden and Shulman, 2002; Emanuela et al., 2012). A HF diet can promote insulin resistance by elevating proteins levels of the negative regulator, PTP1B, which impairs hepatic insulin signalling (Zabolotny et al., 2008). In the liver, insulin signal transduction suppresses hepatic glucose production through the inhibition of gluconeogenesis (Saltiel and Kahn, 2001). The hepatic insulin signalling cascade results in the inhibition of FOXO1, a transcription

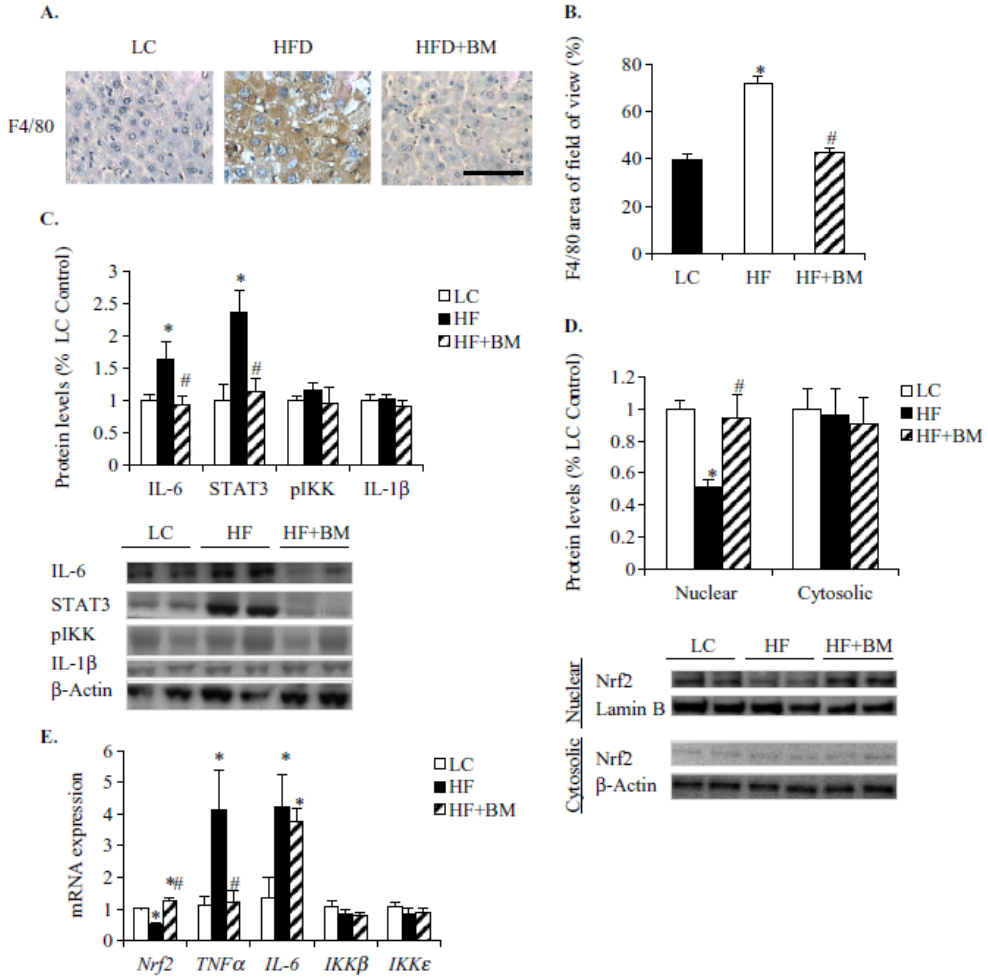

Fig. 4. Effect of chronic administration of bardoxolone methyl (BM) on macrophage infiltration (A) and area of F4/80 immunoreactivity (B), inflammatory proteins (C) IL-6, STAT3, pIKK, and IL-1β, (D) nuclear and cytosolic protein expression of Nrf2, and genes (E) *Nrf2*, *TNFα*, *IL-6*, *IKKβ* and *IKKε*, in the livers of mice (n = 7 per group) fed a high fat (HF) diet for 21 weeks. *, $p = <0.05$ vs. lab chow (LC) group, #, $p = <0.05$ vs. HF group values are means ± SEM. Scale bar = 50 μm.

factor that promotes the expression of gluconeogenic genes such as *G6Pase* (Schmoll et al., 2000; Yeagley et al., 2001). Mice lacking the hepatic *FOXO1* gene display reductions in gluconeogenic gene expression, resulting in reduced glucose production and improved glucose clearance (Matsumoto et al., 2007). BDNF is also an important molecule for preventing insulin resistance that increases glucose metabolism, resulting in reduced blood glucose levels (Hariharan et al., 1997; Kuroda et al., 2003). Our study demonstrated that BM treatment in mice fed a HF diet for 21 weeks reduced hepatic PTP1B and FOXO1, and increased BDNF protein levels, which was coupled with a reduction in plasma insulin levels. In addition, the HF diet-induced decreases in *IR* and *IRS-1*, and increase in *G6Pase* mRNA expression was significantly prevented by BM treatment. Overall, our results suggest that the action of BM in preventing HF diet-induced glucose intolerance and insulin resistance was through, at least partially, inhibiting FOXO1/*G6Pase* mediated hepatic glucose production.

Obesity from a HF diet is known to result in the accumulation of fat into the liver (Marchesini et al., 2001). A HF diet results in an increase in the expression of the hepatic lipogenic genes, *ACC*, *FAS* and *SCD1* and fat accumulation in the livers of mice fed a HF diet (Choi et al., 2014). Moreover, hamsters fed a HF diet have an increase in hepatic lipidemia that is coupled with a decrease in mRNA expression of the β fatty oxidation gene, *ACOX* (Choi et al., 2013). We found that chronic HF diet feeding resulted in an accumulation of fat and hepatocyte injury in the liver and was associated with an increase in *SCD1* and *FAS*, and a decrease in *ACOX* genes. These alterations were prevented by BM administration. These results suggest that HF diet-induce fat accumulation in the liver and associated decreases in a β oxidation gene can be prevented by BM administration.

Obesity-induced fat accumulation is associated with the infiltration of macrophages into adipose tissue, where they promote the release of pro-inflammatory cytokines such as TNFα and IL-6 (Wellen and Hotamisligil, 2005). IL-6 has been found to increase STAT3 levels to promote inflammatory signalling in human mammary epithelial (hTERT-HME1) cells (Yang et al., 2007). We found that there was increased macrophage infiltration, coupled with an increase of the proteins IL-6, and STAT3, and the *IL-6* and *TNFα* genes in the livers of mice chronically fed a HF diet. Furthermore, our results demonstrated that HF diet-induced macrophage infiltration, along with IL-6 and STAT3 protein levels and *TNFα* mRNA expression, could be prevented by BM administration. However, although BM prevented HF diet induced increases in IL-6 protein expression, it failed to prevent HF diet induced increases in *IL-6* mRNA expression, attributed to the post transcription of IL-6. One of the possible mechanisms for the anti-inflammatory effects of BM in the liver includes preventing the activation of IL-6, resulting in reduced STAT3 and preventing *TNFα* mRNA expression which all contribute to attenuating the pro-inflammatory response.

BM has been reported to be one of the most potent known activators of Nrf2 in several peripheral tissues including the eyes and kidneys (Camer et al., 2014). In the livers of mice, Nrf2 activation causes reduced expression of the inflammatory cytokines, TNFα and IL-6 (Liu et al., 2013; Wang et al., 2013). Nrf2 deletion is associated

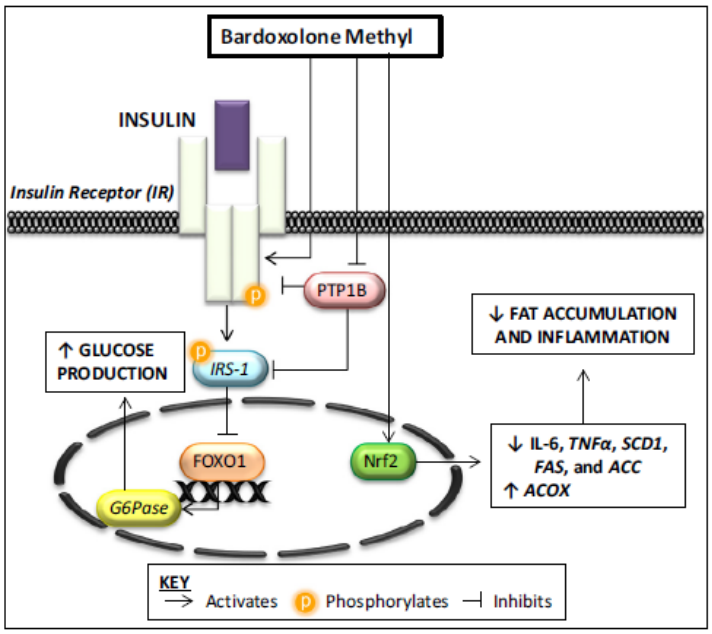

Fig. 5. A proposed model of molecular targets of bardoxolone methyl in preventing HF diet-induced insulin resistance and the development of hepatic steatosis.

with increased liver weight gain, and hepatic steatosis in mice fed a HF diet (Wang et al., 2013). Furthermore, mice deficient in Nrf2 and fed a HF diet have been reported to show rapid development of hepatic steatosis and associated increases in the fatty acid lipogenic genes *ACC*, *FAS* and *SCD1* (Okada et al., 2013) and reduction in the β oxidation gene, *ACOX* (Tanaka et al., 2012). In addition, HF diet-induced hepatic steatosis in mice can be improved through regulation of Nrf2 (Yang et al., 2014). In our study, BM administration prevented HF diet-induced decreases in Nrf2 protein levels in the nucleus and *Nrf2* gene expression. This suggests the ability of BM to prevent HF diet induced elevations in pro-inflammatory signalling molecules and fat accumulation in the liver may be due to its ability to regulate the expression of the *Nrf2* gene and Nrf2 nuclear protein levels.

In conclusion, our findings suggest that chronic supplementation with BM may play an important role in preventing the actions of a HF diet in the development of inflammation, insulin resistance and hepatic steatosis in mice. A proposed model of the potential molecular mechanisms targeted by BM in mice fed a HF diet is presented in Fig. 5. Since obesity-induced inflammation and insulin resistance have been implicated in the progression of liver disease, BM may have beneficial effects in preventing the progression of HF diet-induced liver steatosis. With further research and eventual human clinical trials, the possibility of using BM for the prevention of insulin resistance and associated development of hepatic steatosis appears promising.

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Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.mce.2015.05.018.

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Chapter Five

Bardoxolone methyl prevents the development and progression of cardiac and renal pathophysiologies in mice fed a high-fat diet

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5.1 Author Contributions

D.Camer was a designer of this study, performed all of the experiments, analysed all the data, and wrote the first draft of the manuscript, which all authors reviewed and approved for publication.

5.2 Collaborator Statement

We hereby declare that the statement in section 5.1 pertaining to the contributions of D.Camer is correct.

Dr Yinghua Yu

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Dr Hongqin Wang

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Bardoxolone methyl prevents the development and progression of cardiac and renal pathophysiologies in mice fed a high-fat diet

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Abstract

Obesity caused by consumption of a high-fat (HF) diet is a major risk factor for the development of associated complications, such as heart and kidney failure. A novel semi-synthetic triterpenoid, bardoxolone methyl (BM) was administrated to mice fed a high-fat (HF) diet for 21 weeks to determine if it would prevent the development of obesity-associated cardiac and renal pathophysiologies. Twelve week old male C57BL/6J mice were fed a lab chow (LC), HF (40% fat), or a HF diet supplemented with 10 mg/kg/day BM in drinking water. After 21 weeks, the left ventricles of hearts and cortex of kidneys of mice were collected for analysis. Inflammatory and endothelin signalling molecules were examined in heart and kidney tissue using immunohistochemistry and RT-PCR. Histological analysis revealed that BM prevented HF diet-induced development of structural changes in the heart and kidneys. BM prevented HF diet-induced decreases in myocyte number in cardiac tissue and renal corpuscle hypertrophy in the kidney. Furthermore, in both the hearts and kidneys of mice fed a HF diet, BM administration prevented HF diet-induced increases in fat accumulation, macrophage infiltration and *TNFα* gene expression. These findings suggest that BM prevents HF diet-induced developments of cardiac and renal pathophysiologies in mice fed a chronic HF diet by preventing inflammation. Moreover, these results suggest that BM has the potential as a novel therapeutic for preventing obesity-induced cardiac and renal pathophysiologies

1. Introduction

Obesity caused by the consumption of a high-fat (HF) diet increases the risk of cardiorenal diseases. Cardiovascular disease is the leading cause of death worldwide, with the incidence expected to rise from 17.3 million per year in 2008 to over 23.6 million per year by 2030 (Mozaffarian, Benjamin et al. 2015). There is increasing evidence that obese individuals have an increased risk of developing cardiovascular disease (Kenchaiah, Evans et al. 2002). In addition, there is direct evidence that obesity from a HF diet can cause kidney injury, which also increases the associated cardiovascular disease risk (Prasad 2014). Therefore, there is an urgent need to find suitable therapeutics that can prevent HF diet-induced obesity-associated complications to the heart and kidney, in order to reduce the incidence of global mortality from cardiorenal disease.

The endothelin system has been suggested to play an important role in the development of cardiovascular pathophysiologies. In the heart, endothelin 1 (ET-1) acts through two receptors, endothelin receptor type a (ET_A) and endothelin receptor type b (ET_B). The key endothelin system molecules ET-1, ET_A and ET_B play a role in vasoconstriction, with ET_B also having an additional role in vasodilation (Kedzierski and Yanagisawa 2001). In the cardiac muscle, ET-1 activates ET_A which results in the promotion of cardiac hypertrophy leading to subsequent heart failure (Nasser and El-Mas 2014). Previous studies have demonstrated that there is therapeutic potential in targeting the endothelin system with ET_A or combined ET_A/ET_B antagonists in patients with congestive heart failure (Krum, Viskoper et al. 1998, Nakov, Pfarr et al. 2002). However, it is important to note that in the kidneys the endothelin pathway plays several important roles including the regulation of sodium and water homeostasis and renal blood flow (Kohan 2006). Therefore, over-suppression of the endothelin pathway by antagonistic drugs may lead to other complications in the kidneys such as fluid retention, which if not addressed can also lead to heart

failure (Kohan 2006). Therefore, the development of therapeutics that appropriately targets the endothelin pathway in the heart and kidneys is warranted, in order to prevent obesity-associated cardiovascular disease and renal failure.

Obesity from HF diet is known to result in the development of fat accumulation in peripheral organs, such as the heart and kidneys (Montani, Carroll et al. 2004). Furthermore, peripheral fat accumulation is associated with macrophage infiltration into adipose tissue, which promotes the release of proinflammatory cytokines including tumour necrosis factor alpha (TNFα) (Wellen and Hotamisligil 2005). In a recent study, significantly higher levels of inflammatory markers, including TNFα, were found in the cardiac tissue of Tibetan mini pigs as a result of being fed a HF diet for 24 weeks (Yongming, Zhaowei et al. 2015). Furthermore, rats fed a HF diet for 10 weeks demonstrated increased TNFα levels in the cortex of their kidneys (Elmarakby and Imig 2010). Therefore, novel pharmaceuticals that attenuate TNFα levels may provide a potential therapy for preventing obesity-induced inflammation and tissue damage such as to the heart and kidneys.

In recent years, BM has been extensively studied in both preclinical rodent studies and human clinical trials, and shows promise for the treatment of renal diseases such as chronic kidney disease, and colitis-induced colon cancer due to its anti-inflammatory effects (Pergola, Krauth et al. 2011, Pergola, Raskin et al. 2011, de Zeeuw, Akizawa et al. 2013, Camer, Yu et al. 2014, Choi, Kim et al. 2014). Specifically, studies have demonstrated that BM can reduce inflammation induced by modulating TNFα levels in rodents fed a HF diet (Saha, Reddy et al. 2010, Dinh, Szabo et al. 2015). In addition, our previous studies have highlighted BM as a potential novel therapeutic for preventing HF diet-induced obesity, visceral fat accumulation, and associated development of insulin resistance, hepatic steatosis and cognitive deficits (Camer, Yu et al. 2015, Camer, Yu et al. 2015, Dinh, Szabo et al. 2015). However these positive findings were overshadowed by the recently terminated phase III human clinical trial where there were adverse

cardiovascular events seen in patients with advanced chronic kidney disease treated with BM (de Zeeuw, Akizawa et al. 2013). The mechanisms contributing to these adverse events in the clinical trial were speculated to be via the modulation of the endothelin pathway (Chin, Reisman et al. 2014). However, this pathway was not investigated in the heart tissue (Camer and Huang 2014) and in the kidney following chronic BM treatment, suggesting that further investigation into this drug was vital. In addition, the therapeutic effects of BM treatment on the hearts and kidneys of mice fed a chronic HF diet have not been examined previously.

Here, we provide the first evidence that oral administration of BM prevents HF diet-induced cardiac hypertrophy in mice fed a chronic HF diet. In addition, the development of HF diet-induced kidney pathophysiologies was prevented by BM administration. Specifically, BM administration prevented HF diet-induced macrophage infiltration and elevation of *TNFα* gene expression in the heart and kidneys of mice fed a HF diet. Furthermore, BM treatment suppressed endothelin signalling molecules in the kidney, but elevated expression of endothelin signalling molecules in the heart. These findings indicate the potential of BM as a future therapeutic for the prevention of obesity-related complications, such as cardiac hypertrophy and chronic kidney disease.

2. Materials and Methods

2.1 Animals and HF diet-induced obesity model

Twelve week old C57BL/6J male mice were purchased from the Animal Resource Centre (Perth, Western Australia) and kept in the animal research facility at the University of Wollongong. The experiments were performed in accordance with the *Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.* All procedures were approved by the Animal Ethics Committee, University of Wollongong, Wollongong, Australia (AE 12/15). Mice were housed in environmentally controlled conditions at a constant temperature of 22 °C with a 12 hour light/dark

cycle. Following 1 week of acclimatisation, mice were randomly divided into 3 groups (n=7 per group). For the next 21 weeks one group of mice were fed a lab chow (LC) diet (5% of energy as fat; Vella Stock Feeds, Doonside, New South Wales, Australia), and the other two groups a HF diet (40% of energy as fat; SF11-095, Specialty Feeds, Glen Forrest, Western Australia). Mice in the treatment group were fed a HF diet for 21 weeks, along with a daily oral dose of BM (10 mg/kg) in their drinking water. The dose was chosen according to a previous study (Wu, Liu et al. 2014). Body weights of mice were measured weekly for the duration of the experiment (Final average body weight after 21 weeks: LC, 27.15g; HF, 40.84g; HF+BM, 28.13g).

2.2 Tissue collection

Mice were euthanised (n=7 per group) at week 21 of the experiment. The kidneys and heart were dissected from each mouse. The full hearts were weighed before the apex was cut and place in 10% formalin. The right kidneys of each mouse were cut in half before the inferior portion was place into 10% formalin. The remaining heart and kidney tissue were snap frozen in liquid nitrogen, and stored at -80 °C until use.

2.3 Microdissection

Frozen heart and kidney tissue were cut into 10 µm sections with a cryostat at -18 °C before being mounted on Polylysine™ microscope slides for histological staining. Specifically, the apex of the hearts and the superior portion of the cortex of the kidney were sectioned. The left ventricle of each mouse heart and inferior portion of the kidney cortex were micro-dissected from 500 µm thick frozen sections, and collected for RT-PCR. Kidney and heart tissue were both stored at a temperature of -80 °C until use.

2.4 Oil Red O staining

Oil Red O staining was used to examine lipid accumulation in the heart and kidneys as described previously (Kudo, Tamagawa et al. 2007, Camer, Yu et al. 2015). Briefly, frozen heart and kidney

sections (10 μm) were stained with 0.5% Oil Red O (Sigma-Aldrich) for 15 minutes and then washed. Three fields from three sections collected from each mouse were viewed under a Leica microscope, and digital photographs were captured. Image J software (http://imagej.nih.gov/ij/download.html) was used to quantify staining, which corresponded to the percentage of stained lipid droplets on an area of each slide (Mehlem, Hagberg et al. 2013).

2.5 Haematoxylin and Eosin (H&E) staining

Briefly, frozen kidney and heart sections (10 μm) were stained with Haematoxylin and Eosin (POCD Scientific, Artamon, Australia) for 30 seconds each. Three fields from three sections of each mouse were viewed under a Leica microscope and digital photographs captured. The histological parameters of glomerular and Bowman's capsule hypertrophy in the kidneys were calculated according to the methods described by previous studies (Al-Douahji, Brugarolas et al. 1999, Henegar, Bigler et al. 2001). In the heart tissue, myocytes were measured quantitatively using the software, Image J according to our previous study (Camer, Yu et al. 2015, Dinh, Szabo et al. 2015).

2.6 Immunohistochemistry

Immunohistochemistry was performed as described previously (Camer, Yu et al. 2015, Dinh, Szabo et al. 2015). Briefly, heart and kidney sections fixed in 10% formalin were embedded in paraffin before being sectioned (5 μm) onto Polylysine™ slides. Slides were incubated overnight at 4 °C with anti-rabbit F4/80, anti-goat ET-1, anti-goat ET_B, or anti-rabbit ET_A primary antibody (1:150 Santa Cruz Biotechnology, Dallas, TX) diluted in blocking buffer. Samples were then incubated consecutively at room temperature for 30 minutes with their respective secondary antibody (1:150 Santa Cruz Biotechnology, TX) and then streptavidin-HRP polymer conjugate (1:1000 2438, Sigma-Aldrich Pty Ltd, Sydney, Australia). A DAB peroxidise substrate kit (4100, Vector Laboratories Inc, Burlingame, CA) was used for the development of the stained sections

before counterstaining with H&E (POCD Scientific, Artarmon, Australia). Three fields from three sections of each mouse were viewed under a Leica microscope and digital photographs captured. Image J software was used to quantify the area of F4/80, ET-1, ET_A, or ET_B staining in heart and kidney tissue on each slide.

2.7 RNA isolation and RT-PCR

Total RNA was extracted from dissected mouse heart and kidneys using the Aurum total RNA mini kit (Bio-Rad Laboratories, Hercules, CA) before being reversed transcribed to complimentary first strand DNA with a high-capacity cDNA reverse transcription kit (AB Applied Biosystems, California, USA) according to the manufacturer's directions. Quantitative real-time PCR (RT-PCR) was performed using a Light cycler 480 real time PCR system (F.Hoffmann-La Roche Ltd, Switzerland). A 20µl final reaction volume containing cDNA sample and SYBR green I master mix was used for PCR Briefly, amplification was carried out with 45 cycles of 95 °C for 10 seconds, 60 °C for 30 seconds and 72 °C for 30 seconds. The expression of mRNA was normalised to an internal control, GADPH. The degree of mRNA expression was calculated using the comparative threshold cycle value (Ct) method, using the formula $2^{-\Delta\Delta Ct}$ (where ΔΔCt =ΔCt sample - ΔCt reference) as described previously (Camer, Yu et al. 2015, Cheng, Yu et al. 2015). The primers used are listed in Table 1.3.

2.8 Statistics

Data were analysed using the statistical package SPSS 20 (SPSS, Chicago, IL). Data was first tested for normality before differences between mice fed a LC diet, HF diet, or HF diet administered with BM diet were determined by one-way analysis of variance (ANOVA). This was followed by the post hoc Tukey-Kramer honestly significant difference (HSD) test for multiple comparisons among the groups. A p value of <0.05 was considered statistically significant. Values are expressed as mean ± SEM.

3. Results

3.1 Bardoxolone Methyl prevented the development of cardiac hypertrophy, fat accumulation, and inflammation in mice fed a high-fat diet

To assess whether BM treatment can prevent diet-induced cardiac hypertrophy, we analysed heart tissue in mice fed a HF diet for 21 weeks. Following 21 weeks on a HF diet, the hearts of mice had significantly higher weights than mice fed a LC diet (Final heart weight: -20.66%, $p<0.001$, Figure 1A). This increase in heart weight was prevented by oral administration of BM (Final heart weight: -27.04%, $p<0.001$, Figure 1A). However, there were no significant differences in heart to body weight ratios between any of the groups (Figure 1B). We performed haematoxylin and eosin (H&E) and oil red O staining to examine the effects of BM on myocyte number and lipid content in the heart (Figure 1C). Histological examination of mouse hearts revealed that there was a significant decrease in myocyte number and significant increase in cytoplasmic lipid droplets in mice fed a HF diet for 21 weeks compared to LC fed mice (Myocyte count: -37.43%; lipid stained area: +80.12%, $p<0.001$, Figures 1D and 1E). This change in cardiac morphology was significantly attenuated by BM treatment compared to untreated mice fed a HF diet (Myocyte count: +28.11%; lipid stained area: -50.61%, $p<0.001$, Figures 1D and 1E). However, BM treatment failed to revert HF diet-induced alterations in myocyte number and lipid content in cardiac tissue to the levels present in control LC mice (Myocyte count: -12.97 %; lipid stained area: +59.75%, $p<0.001$, Figures 1D and 1E). These results suggest that cardiac hypertrophy and cellular lipid droplet accumulation induced by a HF diet is attenuated with BM treatment.

To investigate the effect of BM on macrophage accumulation in the left ventricle of the heart in HF diet fed mice, we performed immunohistochemistry with an anti-F4/80 antibody. We found that macrophage numbers increased in the left ventricle of HF diet fed mice as indicated by accumulation of F4/80 positive cells (HF vs. LC difference: -53.17%, $p<0.05$, Figure 1F). BM

administration significantly prevented an increase in the numbers of F4/80 positive cells in the left ventricle of the heart in HF diet fed mice (HF vs. BM difference: -67.40%, p<0.05, Figure 1F and 1G). Furthermore, RT-PCR analysis showed a significant increase in *TNFα* and *IKKβ* mRNA expression in the left ventricle of the heart in mice fed a HF diet (HF vs. LC difference: *TNFα*, -38.70%; *IKKβ*, -26.41%; p<0.05, Figure 1H). The alterations in *TNFα* mRNA levels were significantly prevented by BM administration (HF vs. BM difference: -63.12%, p<0.05, Figure 1H). However, BM treatment was unable to prevent HF diet-induced elevations in *IKKβ* mRNA expression (LC vs. BM difference: -25.47%, p<0.05, Figure 1H). No significant differences were found in the mRNA expression of *IL-6*, and *IKKε* between any of the groups. These results suggest that BM prevents the development of HF diet-induced cardiac macrophage infiltration by downregulating proinflammatory signalling molecules in the left ventricle of the heart.

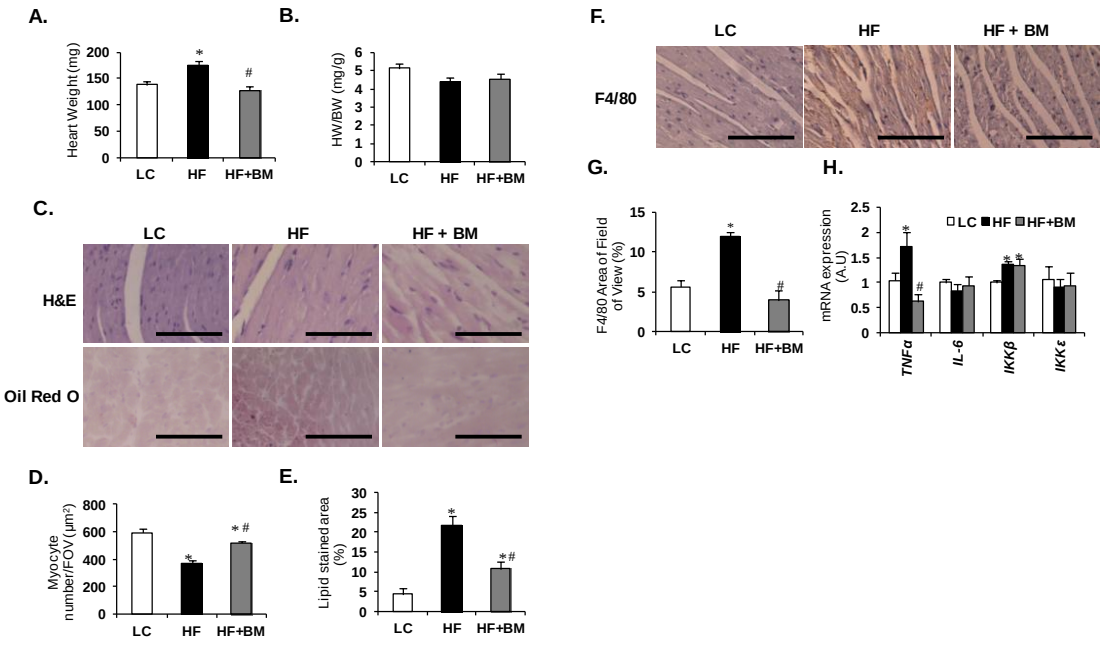

Figure 1. Bardoxolone methyl (BM) attenuated the development of cardiac hypertrophy, lipid accumulation, macrophage infiltration and inflammation in mice fed a chronic high-fat (HF) diet. (A) Heart weights showing significantly lower weights in BM treated mice compared to untreated mice fed a HF diet. (B) Heart to body weight ratio (HW/BW) (C) H&E and Oil Red O staining showing improved cardiac histomorphology and reduced lipid accumulation in mice treated with BM and fed a HF diet. Scale bar= 50μm. (D) Myocyte number per field of view. (E) Cardiac lipid accumulation (F and G) Histology and area of F4/80 immunoreactivity in the hearts of mice. Scale bar= 100μm. (H) RT-PCR analysis of inflammatory genes, *TNFα*, *IL-6*, *IKKβ* and *IKKε*. *, p = <0.05 vs. lab chow (LC) group, #, p= <0.05 vs. HF group values are means ±SEM. (n= 7 mice per group).

3.2 Bardoxolone methyl prevented the development of renal corpuscle hypertrophy, fat accumulation and inflammation in mice fed a high-fat diet

We evaluated whether BM treatment can prevent the development of diet-induced renal hypertrophy and fat accumulation in mice fed a HF diet for 21 weeks through analysis of kidney histomorphology. We performed haematoxylin and eosin (H&E) and oil red O staining to examine the effects of BM on the cellular morphology of the renal corpuscle, and lipid content in the renal cortex (Figure 2A). Compared to LC mice, in mice fed a HF diet for 21 weeks there was a significant increase in thickening of the bowman's capsule and glomerular tuft area in the renal corpuscle that was coupled with a significant increase in cytoplasmic lipid droplets (Bowman's capsule thickening: +91.01%; Glomerular tuft area: +41.96%; lipid stained area: +54.89%, $p<0.001$, Figures 2B-2D). This change in kidney cellular morphology was significantly attenuated by BM treatment compared to untreated mice fed a HF diet (Bowman's capsule thickening: -53.34%; Glomerular tuft area: -36.73%; lipid stained area: -56.85%, $p<0.001$, Figures 2B-2D). However, BM treatment failed to restore the thickness of the Bowman's capsule to normal levels found in LC fed mice (Bowman's capsule thickening: +80.74%, $p<0.001$, Figure 2B). These results suggest that renal corpuscle hypertrophy and cellular lipid droplet accumulation caused by a HF diet are attenuated with BM treatment.

To investigate the effect of BM on macrophage accumulation in the renal cortex of HF diet fed mice, we performed immunohistochemistry with an anti-F4/80 antibody. We found that macrophage numbers increased in the renal cortex of HF diet fed mice as indicated by the accumulation of F4/80 positive cells (HF vs. LC difference: -52.79%, $p<0.05$, Figure 2E). BM administration prevented this increase in F4/80 positive cells (HF vs. BM difference: -46.01%, $p<0.05$, Figure 2E and 2F). Furthermore, RT-PCR analysis showed a significant increase in *TNFα* and *IL-6* mRNA expression in the cortex of kidney tissue in mice fed a HF diet (HF vs. LC

difference: *TNFα*, -66.91%; *IL-6*, -67.79%; $p<0.05$, Figure 2G). The alterations in *TNFα* mRNA levels were prevented by BM administration (HF vs. BM difference: -62.38%, $p<0.05$, Figure 2G). However, there were no significant differences in *IL-6* mRNA levels between BM and the other groups ($p>0.05$, Figure 2G). No significant differences were found in the mRNA expression of *IKKβ* and *IKKε* between any of the groups. These results suggest that BM prevents the development of HF diet-induced renal macrophage infiltration by regulating proinflammatory signalling molecules in the cortex of the kidneys.

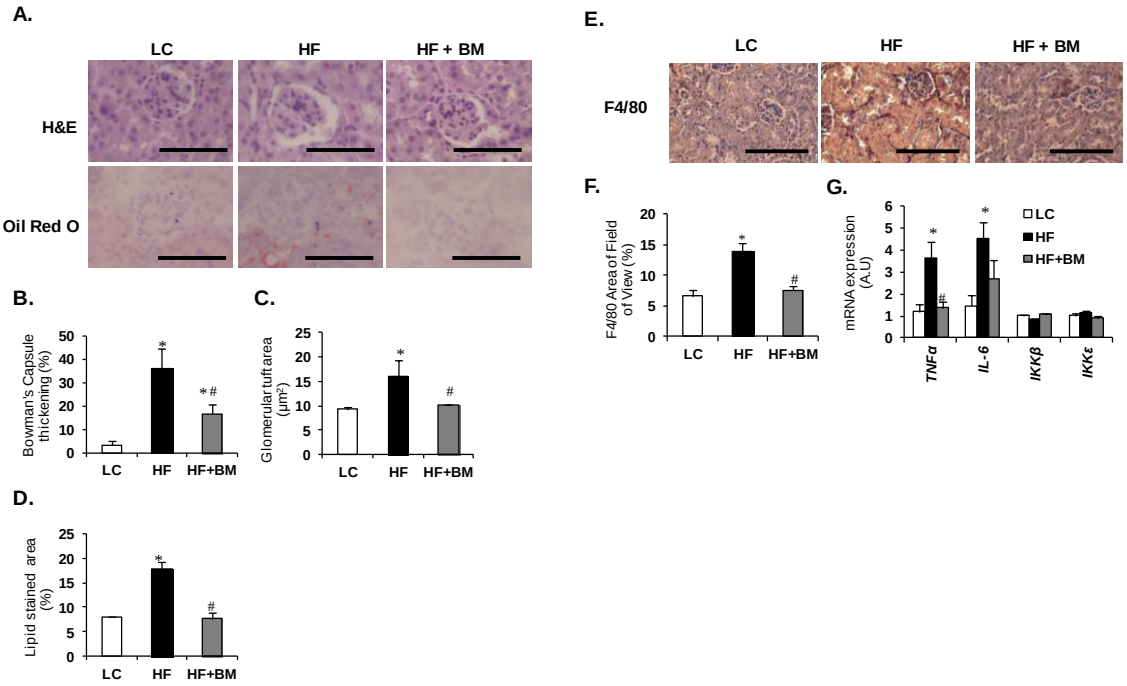

Figure 2. Bardoxolone methyl (BM) attenuated the development of renal corpuscle hypertrophy, lipid accumulation, macrophage infiltration and inflammation in mice fed a chronic high-fat (HF) diet. (A) H&E and Oil Red O staining showing improved renal histomorphology and reduced lipid accumulation in mice treated with BM and fed a HF diet. Scale bar= 50μm. (B and C) Percentage of Bowman's Capsule thickening and Glomerular Tuft Area demonstrating that BM administration prevented renal corpuscle hypertrophy in mice fed a HF diet (D) Renal lipid accumulation (E and F) Histology and area of F4/80 immunoreactivity in the kidneys of mice. Scale bar= 100μm (G) RT-PCR analysis of inflammatory genes, *TNFα*, *IL-6*, *IKKβ* and *IKKε*. *, p = <0.05 vs. lab chow (LC) group, #, p= <0.05 vs. HF group values are means ±SEM. (n= 7 mice per group).

3.3 Bardoxolone methyl treatment failed to restore ET_A protein expression to normal levels and elevated cardiac endothelin signalling genes in mice fed a high-fat diet

Endothelin signalling proteins in the left ventricles of mouse hearts were assessed using immunohistochemistry in order to examine whether BM could prevent HF diet-induced increases

in endothelin signalling. Protein levels of ET_A were significantly elevated in mice fed a HF diet compared to LC diet fed mice (HF vs. LC difference: -93.35%, $p<0.05$, Figure 3A and Table 1). BM treatment failed to restore HF diet-induced elevations in ET_A protein to normal levels present in LC fed mice (BM vs. LC difference: -92.62%, $p<0.05$, Figure 3A and Table 1). There were no differences in ET-1 or ET_B protein levels found between any of the groups ($p>0.05$, Figure 3A and Table 1).

Endothelin signalling gene transcription in the left ventricle of mouse hearts were examined using RT-PCR. Mice fed a HF diet were found to have increased expression of the ET_A gene (HF vs. LC difference: -45.07%, $p<0.05$, Figure 3B), and decreased expression of ET_B gene compared to LC diet fed mice (HF vs. LC difference: -63.04%, $p<0.05$, Figure 3B). BM treatment prevented the HF diet-induced decrease in ET_B gene expression (HF vs. BM difference: -60.62%, $p<0.05$, Figure 3B). However, BM treatment also resulted in a significant increase in *ET-1* gene expression compared to untreated HF diet fed mice (HF vs. BM difference: -56.34%, $p<0.05$, Figure 3B). There were no significant differences in *ET-1* gene expression between LC fed mice and mice fed a HF diet treated with BM ($p>0.05$, Figure 3B). Furthermore, BM administration elevated ET_A gene expression to levels higher than both the LC fed mice and the untreated HF diet fed mice (LC vs. BM difference: -60.72%, $p<0.05$; HF vs. BM difference: -28.49%, $p<0.05$, Figure 3B). These results suggest that BM fails to restore HF diet-induced elevations of ET_A receptor protein levels, and elevates the expression of the endothelin signalling genes, *ET-1*, ET_A, and ET_B in the left ventricles of mice fed a HF diet.

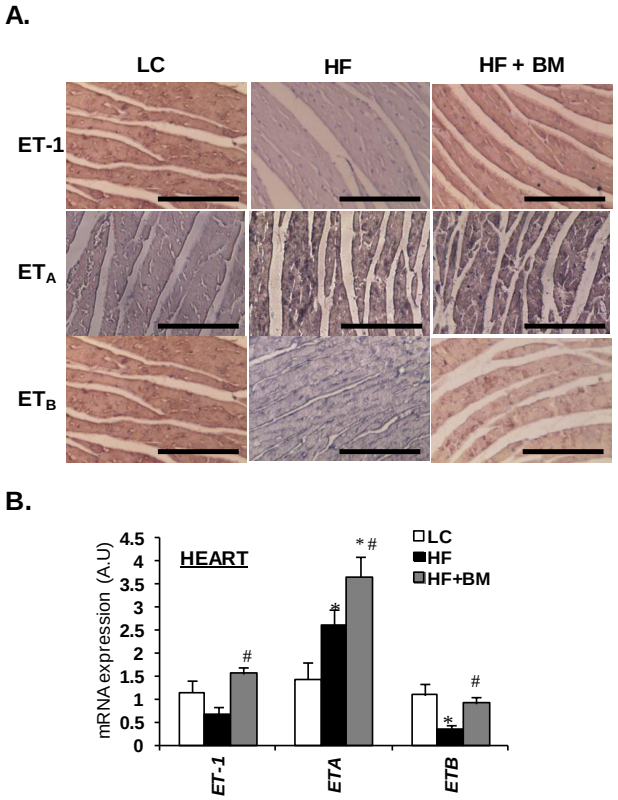

Figure 3. Bardoxolone methyl (BM) elevated cardiac endothelin signalling in mice fed a chronic high-fat (HF) diet. (A) Cardiac ET-1, ET_A and ET_B proteins detected by immunohistochemistry. (B) RT-PCR analysis of cardiac *ET-1*, *ET_A* and *ET_B* genes. *, p = <0.05 vs. lab chow (LC), #, p= <0.05 vs. HF group, values are means ±SEM. Scale bar= 50µm. (n= 7 per group).

Table 1 Endothelin protein levels in mouse heart and kidneys following 21 weeks of LC, HF or HF + BM diet

	Protein	LC	HF	HF+BM	F value	P value
Heart	ET-1	68.0±2.8	54.4±9.7	70.4±3.9	2.101	0.193
	ET_A	0.95±0.5[b]	14.4±3.8[a]	12.9±3.1[a]	7.857	0.013
	ET_B	68.7±3.8	47.9±8.4	59.0±3.4	2.813	0.119
Kidney	ET-1	43.1±7.3	50.8±6.7	25.1±1.9[b]	5.153	0.032
	ET_A	59.6±4.6	59.4±3.3	41.9±8.8	2.824	0.112
	ET_B	53.5±6.0	43.7±6.3	52.8±6.7	0.728	0.512

Values are means ±SEM. LC, lab chow diet, HF, high-fat diet, HF+BM, high-fat diet and bardoxolone methyl treatment. [a]p<0.05 vs LC, [b]p<0.05 vs HF.

3.4 Bardoxolone methyl treatment reduced renal endothelin signalling in mice fed a high-fat diet

Endothelin signalling proteins in the cortex of mouse kidneys were examined using immunohistochemistry in order to assess if BM could prevent HF diet-induced renal dysfunction. There were no significant differences in the protein levels of ET-1 in HF diet fed mice compared to mice fed a LC diet (p>0.05, Figure 4A and Table 1). However, ET-1 protein levels were

significantly reduced in mice treated with BM compared to untreated mice fed a HF diet (HF vs. BM difference: -51.63%, p<0.05, Figure 4A and Table 1). There were no differences in ET_A or ET_B protein levels found between any of the groups (p>0.05, Figure 4A and Table 1).

Furthermore, endothelin signalling genes in the cortex of mouse kidneys were measured using RT-PCR. Mice fed a HF diet were found to have increased expression of the ET_A gene compared to LC diet fed mice (HF vs. LC difference: -49.31%, p<0.05, Figure 4B). This HF diet-induced increase in ET_A gene expression was prevented by BM administration (HF vs. BM difference: -46.70%, p<0.05, Figure 4B). There were no difference in *ET-1* or ET_B gene expression found between any of the groups (p>0.05, Figure 4B). These results suggest that BM prevents HF diet-induced elevations in ET_A gene expression, and significantly reduces ET-1 protein levels in the cortex of the kidneys of mice fed a HF diet.

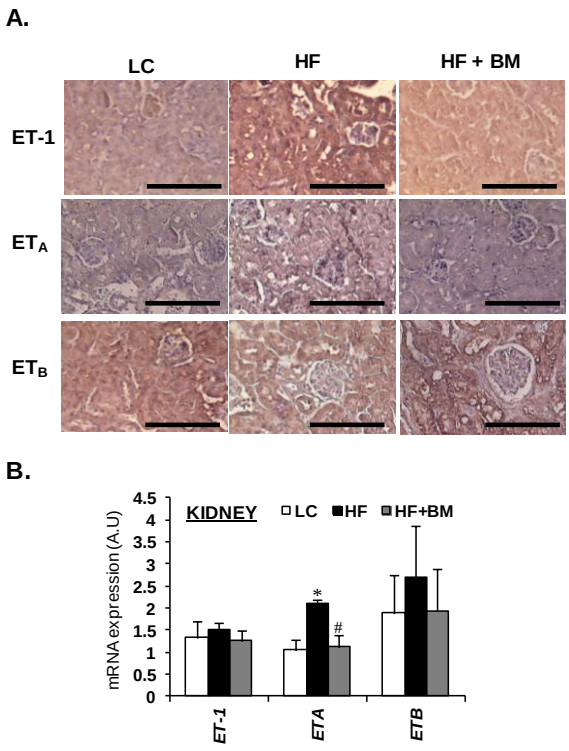

Figure 4. Bardoxolone methyl (BM) reduced renal endothelin signalling in mice fed a chronic high-fat (HF) diet. (A) Renal ET-1, ET_A and ET_B proteins detected by immunohistochemistry (B) RT-PCR analysis of renal *ET-1*, ET_A and ET_B genes. *, p = <0.05 vs. lab chow (LC), #, p= <0.05 vs. HF group, values are means ±SEM. Scale bar= 50µm. (n= 7 per group).

4. Discussion

It is well established that a HF diet can lead to the development of complications in the heart and kidneys, such as cardiac hypertrophy and chronic kidney disease (van Bilsen and Planavila 2014, Hariharan, Vellanki et al. 2015). Rodents fed a chronic HF diet have increased body weight gain, along with structural and functional changes in the kidneys and heart (Deji, Kume et al. 2009, Dhahri, Drolet et al. 2014). Previously, BM has shown promise in treating chronic kidney disease in phases I and II of human clinical trials via anti-inflammatory mechanisms (Pergola, Krauth et al. 2011, Pergola, Raskin et al. 2011). Furthermore, the therapeutic benefits of BM have been demonstrated in HF diet-induced obese animal models such as preventing HF diet-induced visceral fat accumulation, insulin resistance, hepatic steatosis and recognition memory decline in mice (Camer, Yu et al. 2015, Camer, Yu et al. 2015, Dinh, Szabo et al. 2015). The effects of chronic BM administration on the prevention of renal and cardiac pathophysiologies in mice fed a chronic HF diet have not been examined previously. In this present study, we found that feeding a chronic HF diet to mice induces fat accumulation, structural changes and inflammation in the heart and kidneys, which was attenuated by BM administration. These results suggest that BM has the potential to prevent the development of renal and cardiac complications of HF diet-induced obesity.

There is compelling evidence that overweight or obese individuals have an increased risk of heart failure due to left ventricular cardiac hypertrophy (Levy, Garrison et al. 1990, Russo, Jin et al. 2011, Barton, Baretella et al. 2012). Cardiac hypertrophy is characterised by an increase in myocyte size (Chien, Knowlton et al. 1991) and the activation of ET-1 (Huang, Zhang et al. 2011). Along with an increase in heart weight, Zucker fatty rats have an increase in ET-1, ET_A and ET_B gene expression in the left ventricle of the heart (Huang, Yang et al. 2005). In addition, mice fed a HF diet for 10 weeks have significantly elevated cardiac mRNA expression of ET-1, ET_A and ET_B

genes (Catar, Muller et al. 2014). However, in dogs with congestive heart failure, inhibition of ET_B by an antagonist resulted in increased cardiac pressure and decreased cardiac output, suggesting that the vasodilative actions of ET_B are functionally more important than their vasoconstrictive actions (Wada, Tsutamoto et al. 1997). In our study, BM administration prevented HF diet-induced increases in myocyte size in mice, which was indicated by an increase in myocyte number. However, BM did not prevent HF diet-induced increases in ET_A protein expression and worsened HF diet-induced increases in ET_A and ET-1 gene expression. Despite this, BM prevented HF diet-induced decreases in the expression of the ET_B gene. These results suggest that the therapeutic effects of BM on preventing HF diet-induced cardiac hypertrophy may be as a result of targeting the vasodilative functions of ET_B, or a mechanism other than the endothelin pathway in the heart, such as inflammation.

Previous studies have demonstrated that obesity can lead to the development of significant structural and functional changes to the kidneys that can progress to renal or even heart failure (Weisinger, Kempson et al. 1974, Hall, Brands et al. 1993). Obese dogs fed a HF diet were found to have an expansion in Bowman's capsule area and glomerular tuft area in their kidneys compared to lean dogs (Henegar, Bigler et al. 2001). Our study demonstrated that chronic BM administration can prevent the expansion in Bowman's capsule area and glomerular tuft area induced by HF diet in obese mice, suggesting BM has potential to prevent obesity associated kidney damage. Along with alterations in the structure and function of the kidneys, obesity induced by a chronic HF diet is associated with activation of the renal endothelin pathway (Barton 2014). For example, mice fed a chronic HF diet develop obesity, which is coupled with an increase in mRNA expression of ET_A and increased protein expression of ET-1 in the kidneys (Zhang, d'Uscio et al. 2001). BM has been found to suppress the renal endothelin pathway in the kidneys of rodents induced with chronic kidney disease by reducing the protein expression of ET_A

(Chin, Reisman et al. 2014). In this study, we also found that chronic BM administration prevented HF diet-induced increases in mRNA expression of ET_A. In addition, our study demonstrated that BM treatment significantly decreased the protein expression of ET-1. Our results support findings from previous research that demonstrate that BM suppresses renal endothelin signalling molecules. Furthermore, our results add additional knowledge that this drug can also prevent HF diet induced increases in molecules involved in modulating the endothelin signalling pathway.

There is extensive scientific evidence that BM can improve kidney function by inhibiting inflammation in a number of rodent studies and human clinical trials (Pergola, Krauth et al. 2011, Wu, Wang et al. 2011, Ruiz, Pergola et al. 2013). However, no study has investigated the effects of BM on the heart, and thus we investigated the potential preventative effects of BM on inflammation in the hearts and kidneys of mice fed a chronic HF diet. Along with increased fat accumulation, we found that there was elevated macrophage infiltration that was coupled with an increase in the proinflammatory *TNFα* gene in both the left ventricle of the heart and the cortex of the kidneys of mice fed a chronic HF diet. Furthermore, our results demonstrated that chronic BM treatment prevented HF diet-induced fat accumulation, macrophage infiltration and elevated *TNFα* gene expression in the left ventricular area of the heart and cortex of the kidneys of mice. A possible mechanism for these anti-inflammatory effects of BM in these regions of the heart and kidneys includes preventing *TNFα* gene expression and macrophage infiltration, resulting in the attenuation of the proinflammatory response and organ fat accumulation.

In conclusion, our findings suggest that chronic supplementation with BM can prevent HF diet-induced development of cardiac and renal pathophysiologies in mice. Since obesity-induced peripheral fat accumulation and inflammation has been implicated in the progression of heart failure, BM may have beneficial effects in preventing the progression of HF diet-induced cardiac

and renal hypertrophy. With further research and human clinical trials, the possibility of using BM for the prevention of obesity-induced development of renal and cardiac pathophysiologies appears promising.

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Chapter Six

6.1 OVERALL DISCUSSION AND CONCLUSIONS

The present series of studies demonstrated that bardoxolone methyl (BM) administration at a dosage of 10 mg/kg daily in drinking water prevented diet-induced obesity, type 2 diabetes and recognition memory decline in mice fed a chronic HF diet for 21 weeks. In addition, these studies show that BM treatment can target multiple tissues through its ability to prevent obesity-induced pathophysiologies of peripheral organs such as the liver, heart, and kidneys in the same animals. Overall, these studies identify BM as a potential novel therapeutic for preventing the development and progression of HF diet-induced obesity and associated complications. These findings from this thesis also contribute novel data towards understanding the mechanisms underlying the therapeutic effects of BM in preventing diet-induced obesity, type 2 diabetes, and associated complications in the brain, liver, heart, and kidneys of mice fed a chronic HF diet. This chapter will provide a general discussion of the findings and the potential mechanisms underlying the therapeutic properties of BM based on its effects on intracellular signalling pathways in the target cell. A detailed discussion of each study has been included at the end of Chapters 2-5.

6.1.1 Proposed mechanisms of BM in preventing HF diet-induced obesity and associated complications

In Chapter 2, it was found that BM treatment prevented HF diet-induced obesity, increased energy intake, and peripheral fat accumulation in male C57BL/6J mice fed a HF diet for 21 weeks. It has previously been reported that BM treatment in a phase II human clinical trial of patients with chronic kidney disease and type 2 diabetes caused dramatic weight loss where treated patients lost 5-10kg more than the placebo group over a 24 week period (Pergola, Raskin et al. 2011). However, the possible mechanisms explaining this effect were unclear. Therefore,

Chapter 2 was the first to uncover the mechanisms behind BM's anorexigenic effect in a chronic HF diet-induced obesity prevention study. Since BM administration prevented HF diet-induced increases in energy intake, it was proposed that this compound regulates negative energy balance by targeting the hypothalamus, an important brain region for maintaining energy homeostasis. As discussed in Chapters 1 and 2, Leptin-JAK2-Akt-FOXO1 signalling in both the mediobasal and paraventricular nuclei regions of the hypothalamus is essential for the regulation of energy balance (Bell, Bhatnagar et al. 2000, Morton, Gelling et al. 2005, Munzberg and Myers 2005, Kim, Pak et al. 2006, Metlakunta, Sahu et al. 2008, Plum, Lin et al. 2009, Roman, Reis et al. 2010). In obesity, downstream hypothalamic leptin signalling is largely impaired due to accentuated activation of the negative regulators, PTP1B and AMPK, which inhibit JAK2 activation, resulting in leptin resistance (Zabolotny, Bence-Hanulec et al. 2002, Andersson, Filipsson et al. 2004, Minokoshi, Alquier et al. 2004, Bence, Delibegovic et al. 2006, Bence, Delibegovic et al. 2006, Lam, Covey et al. 2006, Zhang, Zhang et al. 2008, White, Whittington et al. 2009, Lu, Wu et al. 2011, Su, Jiang et al. 2012). In Chapter 2, it was confirmed that mice fed a chronic HF diet were obese and leptin resistant, which was prevented by BM administration. Importantly, these results also demonstrated that BM treatment prevents HF diet-induced impairments to downstream leptin signal transduction by maintaining phosphorylation of JAK2, Akt and FOXO1 and preventing increases in PTP1B and phosphorylation of AMPK proteins in the mediobasal and paraventricular nuclei regions of the hypothalamus. Collectively, these results of Chapter 2 show for the first time that BM prevents HF diet-induced impairments to energy balance through modulating downstream leptin signalling in the mediobasal and paraventricular nuclei regions of the hypothalamus.

Along with the development of obesity, several studies have demonstrated that a HF diet can cause cognitive decline, including impairments in recognition memory (Kanoski, Meisel et al.

2007, Valladolid-Acebes, Stucchi et al. 2011, Heyward, Walton et al. 2012, Carey, Gomes et al. 2014). As discussed in Chapter 1, downstream BDNF-pTrkB-Akt signalling in the PFC and hippocampus promotes neuronal plasticity and neurogenesis, which are both important for learning and memory (Kanoski, Meisel et al. 2007, Cunha, Brambilla et al. 2010, Sakata, Martinowich et al. 2013). Mice fed a HF diet display decreased BDNF levels in both hippocampus and PFC, which is coupled with impaired discrimination learning (Kanoski, Meisel et al. 2007). In a previous study, a derivative of BM, CDDO-MA, improved spatial memory and reduced inflammation in the hippocampus in a mouse model of Alzheimer's disease (Dumont, Wille et al. 2009). However, the results from this current study were the first to investigate the effects of BM on cognition in a HF diet-fed mouse model. The novel data from Chapter 3 found that BM prevented deficits in recognition memory in mice fed a chronic HF diet. This result was coupled with increased BDNF levels along with protein phosphorylation of downstream signalling molecules, TrkB and Akt in the hippocampus and PFC. Overall, the findings from Chapter 3 suggests that the actions of BM on the downstream BDNF signalling cascade contributed to improved neuronal plasticity in the hippocampus and PFC of mice fed a HF diet, which further contributed to an improvement in recognition memory. However, only BDNF levels in the PFC were positively correlated to recognition memory, suggesting that BDNF signalling in the PFC is important for recognition memory.

As reviewed in Chapter 1, HF diet-induced obesity largely increases the risk of developing type 2 diabetes. A HF diet can promote insulin resistance by elevating protein levels of the negative regulator, PTP1B, which impairs downstream IR-IRS-pAkt-FOXO1 hepatic insulin signalling (Schmoll, Walker et al. 2000, Saltiel and Kahn 2001, Yeagley, Guo et al. 2001, Matsumoto, Pocai et al. 2007, Zabolotny, Kim et al. 2008). BM has recently received considerable attention because of its blood glucose lowering effects in an acute diet-induced obese mouse model (Saha, Reddy

et al. 2010). However, my findings from Chapter 4 were the first to demonstrate that BM treatment prevents the development of diet-induced insulin resistance in mice fed a chronic HF diet. Furthermore, these novel data shed further light into the potential mechanisms behind BM's effects in regulating blood glucose levels. In this study, BM treatment in mice fed a HF diet for 21 weeks reduced hepatic PTP1B and FOXO1, and increased BDNF protein levels, which was coupled with a reduction in plasma insulin levels. In addition, the HF diet-induced decreases in *IR* and *IRS-1*, and increase in *G6Pase* mRNA expression was significantly prevented by BM treatment. Overall, these results from Chapter 4 suggest that the action of BM in preventing HF diet-induced glucose intolerance and insulin resistance was through, at least partially, inhibiting FOXO1/*G6Pase* mediated hepatic glucose production.

As discussed in Chapter 1 and in our published review paper (Appendix 1.1), BM has been reported to be one of the most potent known activators of Nrf2 in several peripheral tissues including the eyes and kidneys (Camer, Yu et al. 2014). In the livers of mice, Nrf2 activation causes reduced expression of the inflammatory cytokines, TNFα and IL-6 (Liu, Wu et al. 2013, Wang, Cui et al. 2013). Nrf2 deletion is associated with increased liver weight gain, and hepatic steatosis in mice fed a HF diet (Wang, Cui et al. 2013). Furthermore, mice deficient in Nrf2 and fed a HF diet have been reported to show rapid development of hepatic steatosis (Tanaka, Ikeda et al. 2012, Okada, Warabi et al. 2013). In addition, HF diet-induced hepatic steatosis in mice can be improved through regulation of Nrf2 (Yang, Li et al. 2014). In Chapter 4 of this study, BM administration prevented HF diet-induced increases in fat accumulation and inflammation, and decreases in Nrf2 protein levels in the nucleus and *Nrf2* gene expression in the livers of mice. These findings suggest that the ability of BM to prevent HF diet-induced elevations in proinflammatory signalling molecules and fat accumulation in the liver may be due to its ability to regulate the expression of the *Nrf2* gene and Nrf2 nuclear protein levels.

As reviewed in Chapter 1, it is well established that HF diet-induced obesity increases the risk of developing structural and functional complications in the heart and kidneys, such as cardiac hypertrophy and chronic kidney disease (Weisinger, Kempson et al. 1974, Levy, Garrison et al. 1990, Hall, Brands et al. 1993, Henegar, Bigler et al. 2001, Russo, Jin et al. 2011, Barton, Baretella et al. 2012, van Bilsen and Planavila 2014, Hariharan, Vellanki et al. 2015). Along with alterations in the structure and function of the heart and kidneys, obesity induced by a chronic HF diet is associated with activation of the cardiac and renal endothelin pathway signalling molecules, ET-1, ET_A, and ET_B (Zhang, d'Uscio et al. 2001, Barton 2014, Catar, Muller et al. 2014). However, the function of the endothelin signalling molecule, ET_B in the heart is suggested to have more vasodilative than vasoconstrictive actions (Wada, Tsutamoto et al. 1997). The potential of BM for future use in the clinic has been promising for treating chronic kidney disease in phases I and II of human clinical trials via anti-inflammatory mechanisms (Pergola, Krauth et al. 2011, Pergola, Raskin et al. 2011). However, this positive result was recently overshadowed by the subsequent phase III human clinical trial in patients with type 2 diabetes and stage 4 chronic kidney disease which was terminated due to increased cardiovascular events in BM treated patients (de Zeeuw, Akizawa et al. 2013). The mechanisms behind these adverse effects were speculated to be through the suppression of the renal endothelin pathway in the kidneys of rodents induced with chronic kidney disease (Chin, Reisman et al. 2014). However, this conclusion was based on the investigation of kidney, but not heart tissue. We raised this concern in our published invited perspective highlighting that further investigation of the effects of BM on the endothelin pathway needed to be performed in both kidney and heart tissue as the role of endothelin signalling differs in each tissue (Camer and Huang 2014) (Appendix 1.2). Therefore, it was important to test the effects of BM on endothelin signalling in the heart and kidneys in Chapter 5 of this study. The findings from Chapter 5 on the effects of BM on heart tissue

demonstrated that BM administration prevents increases in myocyte size and renal corpuscle hypertrophy in mice fed a chronic HF diet. However, BM did not prevent HF diet-induced increases in cardiac ET_A protein expression and worsened HF diet-induced increases in ET_A and ET-1 gene expression. Despite this, BM prevented HF diet-induced decreases in the expression of the ET_B gene in the heart. On analysis of kidney tissue, the results from Chapter 5 supported findings from previous research by demonstrating that BM suppresses the renal endothelin signalling protein, ET-1, and gene, *ET_A*. Collectively, the results from Chapter 5 suggest that the therapeutic effects of BM in preventing HF diet-induced cardiac hypertrophy may be as a result of targeting the vasodilative functions of ET_B, or a mechanism other than the endothelin pathway in the heart, such as inflammation. Moreover, in the kidney tissue, these findings add additional knowledge that this drug can also prevent HF diet-induced increases in molecules involved in modulating the renal endothelin signalling pathway, suggesting a mechanism for preventing HF diet-induced renal pathophysiologies.

As discussed in Chapter 1, chronic low grade inflammation is a key characteristic of obesity which promotes neuroinflammation, leading to the development of hypothalamic leptin resistance and impairments to synaptic plasticity in the forebrain (De Souza, Araujo et al. 2005, Sunayama, Tsuruta et al. 2005, Zabolotny, Kim et al. 2008, Lu, Wu et al. 2011, Fuentes, Zimmer et al. 2012, Thaler, Yi et al. 2012, Benzler, Ganjam et al. 2013, Wang, Fu et al. 2013). My findings from Chapter 2 demonstrated that BM administration prevented elevations in the activation of the proinflammatory mediator, JNK, and the proinflammatory cytokines, TNFα and IL-6, in the mediobasal and paraventricular nuclei regions of the hypothalamus of mice fed a chronic HF diet. Moreover, in Chapter 3, BM treatment was found to prevent elevated levels of inflammatory mediators, such as PTP1B in the PFC and hippocampus. Collectively, these results from Chapters 2 and 3 demonstrate the anti-inflammatory effects of BM in preventing

neuroinflammation and associated tissue damage in the hypothalamus, PFC and hippocampus induced by a chronic HF diet.

In addition to uncovering the therapeutic effects of BM on the brain (Chapters 2 and 3), a further novel contribution in this project was the discovery that BM treatment has therapeutic benefits in preventing chronic HF diet-induced development of peripheral tissue damage (Chapters 4 and 5). Several studies have demonstrated that a HF diet leads to the development of fat accumulation, macrophage infiltration, and low grade inflammation in peripheral tissues, including the liver, heart, and kidneys (Marchesini, Brizi et al. 2001, Weisberg, McCann et al. 2003, Xu, Barnes et al. 2003, Wellen and Hotamisligil 2005, Yang, Liao et al. 2007, Deji, Kume et al. 2009, Dhahri, Drolet et al. 2014). There is extensive scientific evidence that BM can improve kidney function by inhibiting inflammation in a number of rodent studies and human clinical trials (Pergola, Krauth et al. 2011, Wu, Wang et al. 2011, Ruiz, Pergola et al. 2013). However, no study has previously investigated the effects of BM on the liver, kidneys, and heart of mice fed a chronic HF diet. My results from Chapters 4 and 5 demonstrated that chronic BM treatment prevented HF diet-induced fat accumulation, macrophage infiltration, and inflammation in the liver, heart, and kidneys of mice. Moreover, in all three of these peripheral organs, elevated gene expression of the proinflammatory cytokine, *TNFα,* was prevented by BM administration in mice fed a chronic HF diet. Taken together these data suggest a possible mechanism for these anti-inflammatory effects of BM in the liver, heart, and kidneys through preventing *TNFα* gene expression and macrophage infiltration, resulting in the attenuation of the proinflammatory response. Overall, the results from the present series of studies suggest that BM treatment prevents HF diet-induced associated tissue damage in the brain, liver, heart, and kidneys through anti-inflammatory mechanisms.

6.1.2 Recommendations for Future Research

Based on the findings of the present series of studies, recommendations for further research are as follows:

1. In Chapter 4 of this thesis, it was found that BM treatment prevented alterations in Nrf2 nuclear protein and *Nrf2* gene expression in the livers of mice fed a HF diet for 21 weeks. Since BM is a known potent activator of Nrf2 (Camer, Yu et al. 2014), it would be interesting to test if similar effects are found in other tissues including the brain, heart, and kidneys in a future study.
2. This study was conducted in male mice only. Therefore, female mice should be tested in the future using the same experimental design to determine if there are gender differences.
3. Since mice were used, I was limited by the amount of molecular analysis I could perform due to the small amount of tissue obtained following microdissection. For example, after dissecting the left ventricle of the mouse heart, I only had enough tissue to test a few parameters using immunohistochemistry and RT-PCR. Therefore, a larger animal should be used in future studies, such as rats, in order to obtain more tissue for investigation of more parameters and in order to perform additional important experimental techniques for protein analysis, such as western blotting.
4. BM has successfully completed phase I and II of human clinical trials for treating CKD in individuals with type 2 diabetes, and phase I clinical trials for the treatment of leukaemia and solid tumours (Liby, Yore et al. 2007), indicating its potential in treating multiple diseases. This thesis has found that BM administration prevents HF diet-induced obesity (Chapter 2), cognitive deficits (Chapter 3), insulin resistance (Chapter 4) and development of pathophysiologies in the liver (Chapter 4), heart, and kidneys (Chapter 5)

in a mouse model. Therefore, future studies should involve a human clinical trial in overweight or obese patients to determine if BM can prevent the development and progression of obesity-induced complications.

5. Collectively, the results of this PhD thesis showed that BM had therapeutic effects in preventing chronic HF-diet-induced obesity and associated complications. Although a previous study has tested the effects of acute BM administration for 2 weeks in obese mice with promising results (Saha, Reddy et al. 2010), a chronic treatment study in rodents should be performed in order to see if obesity and development of associated complications can be reversed through chronic BM treatment.
6. In Chapter 3, my results demonstrated that BM treatment prevented impairments to recognition memory in mice fed a chronic HF diet, which was determined through performing a novel object recognition test. A number of studies have demonstrated that HF diet-induced obesity can also impair other areas of cognition, such as spatial memory (Greenwood and Winocur 1990, Greenwood and Winocur 1996, Heyward, Walton et al. 2012). Therefore, other behavioural tests should be performed in the future, such as a Morris Water Maze test, in order to determine if BM has benefits in other areas of cognition in a HF diet animal model.
7. A recent study found that BM treatment suppressed colitis-induced colon cancer via anti-inflammatory mechanism in mice (Choi, Kim et al. 2014). Since HF diet-induced obesity has been found to be associated with the development of colon polyps (Chen and Huang 2015), analysis of the colons of mice treated with BM and fed a HF diet should be tested in the future.
8. It is well established that obesity and associated metabolic disorders can also be induced by pharmaceutics, such as the antipsychotic drug olanzapine (Deng 2013). Therefore,

BM administration should also be tested, such as in an olanzapine animal model, to determine if it can prevent or treat antipsychotic-induced obesity.

9. Overall, the results from this thesis suggested that the ability of BM to target inflammation, in particular the pro-inflammatory cytokines TNFα and IL-6, played an important role in preventing the development of obesity and the mentioned associated complications. However, there are many cytokines, which have not been investigated in this thesis, that play an important role in anti-inflammatory (eg, IL-4, IL-10, IL-11, IL13) and pro-inflammatory (eg, 1L-1, IL-8) mechanisms (Xu, Barnes et al. 2003). In addition, no previous study has examined the effects of BM treatment on these pro-inflammatory and anti-inflammatory cytokines in HF diet-induced obesity and associated complications Therefore, future studies could investigate whether BM plays an additional role in reducing inflammation through interactions with other pro-inflammatory and anti-inflammatory cytokines.

6.1.3 Conclusion

The results of this PhD thesis have demonstrated that BM treatment can prevent HF diet-induced development of obesity, type 2 diabetes, cognitive deficits, and pathophysiologies of the liver, heart, and kidneys in mice fed a HF diet for 21 weeks. In Chapter 2, chronic BM supplementation significantly prevented food intake, body weight gain, hyperleptinemia, and leptin resistance in mice fed a HF diet for 21 weeks. The mechanisms responsible for this anorexigenic effect of BM were found to be through promoting downstream leptin-JAK2-Akt-FOXO1 signalling and preventing hypothalamic inflammation in both the mediobasal and paraventricular nuclei regions of the hypothalamus. My findings from Chapter 3 also demonstrated that chronic administration of BM prevented impairments to recognition memory in mice fed a HF diet. These cognitive benefits of BM were suggested to be through promotion of downstream BDNF-TrkB-Akt signalling, and

through a decrease in the inflammatory mediator, PTP1B, in the PFC and hippocampus. The present study demonstrates in Chapter 4 that HF diet-induced development of insulin resistance and glucose intolerance could be prevented with BM supplementation. The mechanism of action of BM in this therapeutic effect was found to be through modulation of hepatic insulin-IR-IRS-Akt-FOXO1 signalling resulting in the inhibition of FOXO1/*G6Pase* mediated glucose production. In addition, in Chapters 4 and 5, BM administration was found to prevent HF diet-induced development of peripheral tissue damage in the liver, kidneys, and heart. These findings were found to be anti-inflammatory, with a potential mechanism suggested to be through BM preventing *TNFα* gene expression and macrophage infiltration, leading to the attenuation of the proinflammatory response. Overall, the present series of studies highlighted the anti-inflammatory nature of BM in a variety of tissues. In mice fed a chronic HF diet, BM administration prevented HF diet-induced inflammation in the brain, liver, heart, and kidneys, suggesting a potential overarching anti-inflammatory mechanism in its ability to prevent the development of obesity and associated complications. The present study therefore offers an insight into the mechanisms underpinning the therapeutic properties of BM in preventing the development of HF diet-induced obesity and associated complications. With further research and human clinical trials, the possibility of using BM for the prevention of HF diet-induced obesity, and associated development of type 2 diabetes, cognitive deficits, and pathophysiologies of peripheral tissues such as the liver, kidneys, and heart appears promising, while taking into consideration its potential adverse effects.

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Appendix One

Appendix 1.1 The molecular mechanisms underpinning the therapeutic properties of oleanolic acid, its isomer and derivatives for type 2 diabetes and associated complications

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A1.1.1 Author Contributions

D.Camer reviewed the literature and wrote the first draft of the manuscript, which all authors reviewed and approved for publication.

A1.1.2 Collaborator Statement

We hereby declare that the statement in section A1.1.1 pertaining to the contributions of D.Camer is correct.

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REVIEW

The molecular mechanisms underpinning the therapeutic properties of oleanolic acid, its isomer and derivatives for type 2 diabetes and associated complications

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Recent research has uncovered the molecular mechanisms responsible for the therapeutic properties of oleanolic acid (OA), its isomer ursolic acid (UA), and derivatives. In particular, recent reports have highlighted the benefits of these compounds in the prevention and treatment of type 2 diabetes and associated life-threatening complications, such as nonalcoholic fatty liver disease, nephropathy, retinopathy, and atherosclerosis. The prevalence of type 2 diabetes is of major concern since it is reaching global epidemic levels. Treatments targeting the signaling pathways altered in type 2 diabetes are being actively investigated, and OA and UA in natural and derivative forms are potential candidates to modulate these pathways. We will explore the findings from in vitro and in vivo studies showing that these compounds: (i) improve insulin signaling and reduce hyperglycemia; (ii) reduce oxidative stress by upregulating anti-oxidants and; (iii) reduce inflammation by inhibiting proinflammatory signaling. We will discuss the molecular mechanisms underpinning these therapeutic properties in this review in order to provide a rationale for the future use of OA, UA, and their derivatives for the prevention and treatment of type 2 diabetes and associated comorbidities.

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1 Introduction

Type 2 diabetes has reached epidemic proportions worldwide. Recent predictions indicate that the prevalence of diabetes globally will increase from 285 million in 2010 to 439 million in 2030 [1]. Along with hyperglycemia and reduced insulin sensitivity, other characteristics featured in type 2 diabetes include proinflammation and oxidative stress that contributes to damage to tissue in the liver, kidney, adipose tissue, pancreas, and vasculature. Type 2 diabetes can lead to the progression of a number of potentially life-threatening macrovascular and microvascular complications, including non-alcoholic fatty liver disease, nephropathy, retinopathy, and atherosclerosis [2]. Currently available antidiabetic drugs have limited efficacy and/or safety concerns. Therefore, identifying new medicinal agents, especially extracted from natural products, offers exciting possibilities for future development of successful antidiabetic therapies.

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Abbreviations: **AGE**, advanced glycation end product; **Akt**, Protein kinase B; **AR**, aldose reductase; **ARE**, anti-oxidant response element; **CDDO-Me**, bardoxolone methyl; **CKD**, chronic kidney disease; **FOXO1**, forkhead box protein O1; **GLUT4**, glucose transporter 4; **G6P**, glucose 6 phosphate; **HbA$_{1c}$**, glycated hemoglobin; **HFD**, high-fat diet; **IκB**, nuclear factor kappa B inhibitor alpha; **IKK**, IκB kinase; **IR**, insulin receptor; **IRS**, insulin receptor substrate; **NF-κB**, nuclear factor kappa B; **Nrf2**, nuclear factor like 2; **OA**, oleanolic acid; **PI3K**, phosphoinositide 3 kinase; **PTP1B**, protein tyrosine phosphatase 1B; **ROS**, reactive oxygen species; **SOCS3**, suppressor of cytokine signaling 3; **TNF-α**, tumor necrosis factor alpha; **UA**, ursolic acid

2 Oleanolic acid, its isomer, and derivatives

Both oleanolic acid (OA) and its isomer, ursolic acid (UA), exist widely in nature and can be extracted from fruits, herbs,

and vegetables. OA can be found in olive leaves, olive pomace, mistletoe sprouts, and clove flowers, while UA can be found in apple pomace. A mixture of these two triterpenes can also be found in rosemary leaves [3]. Both OA and UA are pentacyclic triterpenes, which is a group of widespread natural compounds containing six isoprene units, the basic molecular formula C30H48 and with five rings in their skeleton [3, 4]. Recently, OA and UA have received great attention because of their benefits including antihyperglycemic, antihyperlipidemic, anti-inflammatory, and anti-oxidative properties and potential application for the treatment of type 2 diabetes and associated complications [5–12]. Although they differ only in the position of a side chain in their structure, a number of in vitro and animal studies have demonstrated that OA and UA exhibit different degrees of potency in particular functions including their direct binding to insulin signaling molecules such as Protein tyrosine phosphatase 1B (PTP1B) [13–16].

Triterpenoids are structurally similar to steroids and may, like steroids, diffuse freely through cell membranes to interact with intracellular molecular targets. The semisynthetic triterpenoid 2-cyano- 3,12-dioxooleana-1,9(11)-dien-28-oic acid (CDDO) has been developed along with chemically modified derivatives containing various functional groups on rings A and C [17, 18]. These novel compounds are far more potent than natural triterpenoids and can affect signaling pathways in mammalian cells that are associated with detoxification [19], inflammation, and apoptosis [20, 21]. These sites can be accentuated and manipulated through chemical modification of the natural compound into derivative form. Examples of highly potent synthetic OA derivatives are the CDDO derivatives, which are strong anti-oxidant compounds. In particular, the OA CDDO derivative, CDDO-Me (Bardoxolone Methyl) highlights the promising potential of these compounds as it has successfully completed phases I and II of human clinical trials [5, 22].

3 OA and UA lower blood glucose levels by improving insulin Akt signaling

Several studies have demonstrated the ability of OA and UA in normalizing blood glucose levels in rodents with diet-induced obesity or diabetes [6–9, 15, 23]. In particular, in two preventative studies where mice were administered OA or UA at a dosage of 10 mg/kg in conjunction with being fed a high-fat diet (HFD) for 15 weeks, blood glucose levels were significantly lower compared to nontriterpene HFD controls by 37 and 42%, respectively [7, 8]. This demonstrates the strong hypoglycaemic effects of UA and OA and implicates that their effects are caused by targeting insulin signaling and/or glucose producing molecules.

Recent evidence suggests that OA, UA, and a number of their derivatives can improve insulin signaling by enhancing insulin receptor (IR) β subunit phosphorylation and Akt in vitro [24–26]. Insulin regulates glucose homeostasis through binding of its receptor to initiate a signaling cascade; activation and phosphorylation of the insulin receptor substrate (IRS) proteins, and mediation of the phosphatidylinositol 3-kinase-dependent/the protein kinase Akt (phosphoinositide 3 kinase/Protein kinase B [PI3K/Akt]) pathway (Fig. 1). The activation of the Akt pathway can (i) mediate the translocation of glucose transporter 4 (GLUT4) to the plasma membrane, thereby facilitating glucose uptake into adipose tissue, cardiac muscle, and skeletal muscle [27–32], and (ii) inhibit glucose production via gluconeogenesis by glucose 6 phosphate (G6P) and forkhead box protein O1 (FOXO1) in liver.

OA and UA promote glucose uptake from the bloodstream into peripheral tissues through upregulation of GLUT4 [27–32]. In an in vitro study, UA promoted glucose uptake by enhancing the translocation of GLUT4 to the plasma membrane in 3T3-L1 adipocytes [24]. 3T3-L1 adipocytes treated with an OA derivative, NPLC441, had increased GLUT4 mRNA and protein expression compared to untreated cells indicating increased glucose uptake into the cells [26, 33]. A glucose uptake assay in L6 myotubes revealed that a 1 μM dose of an OA derivative increases basal glucose uptake by 40% [26, 33].

Another mechanism of OA and UA in lowering blood glucose is by the reduction of endogenous glucose production via inhibition of gluconeogenesis in the liver. Glucose production via gluconeogenesis can exacerbate hyperglycemic states, and favors the development and progression of type 2 diabetes. Key molecules in the gluconeogenic pathway are G6P and FOXO1 [34, 35]. A total of 0.05% UA supplement in diet decreased glucose-6-phosphatase activity in the livers and significantly elevated the hepatic glycogen content in STZ and high-fat diet-induced diabetic mice [34, 35]. Adding 0.05% OA extracted from dietary wine pomace in HF diet significantly downregulated the mRNA expression of G6P (49%) and FOXO1 (52%) in liver of rats [34, 35].

4 OA and UA inhibit PTP1B resulting in improved insulin signaling

PTP1B has been proposed as a novel target whose inhibition would specifically address insulin resistance. PTP1B is a molecule that negatively regulates insulin signaling [15, 26, 36]. Several in vitro studies have provided evidence that OA, UA, and a number of their derivatives can directly inhibit PTP1B and improve insulin sensitivity [15, 26, 36] (Fig. 1). In particular, a PTP1B inhibition assay concluded that OA and UA adhered to the linear mixed-type inhibition model in their interaction with PTP1B [15]. Interestingly, the binding site of PTP1B targeted by OA and UA was uncovered to be a secondary region, known as site B rather than the typical catalytic binding site A [15]. This suggests that compounds that have high specificity for this region should be developed, such as through modifying OA and UA, to derivative forms in order to achieve strong PTP1B inhibition and subsequent maximal improvement to insulin signal transduction. In addition, the OA and UA derivatives (C-28 addition) were more potent

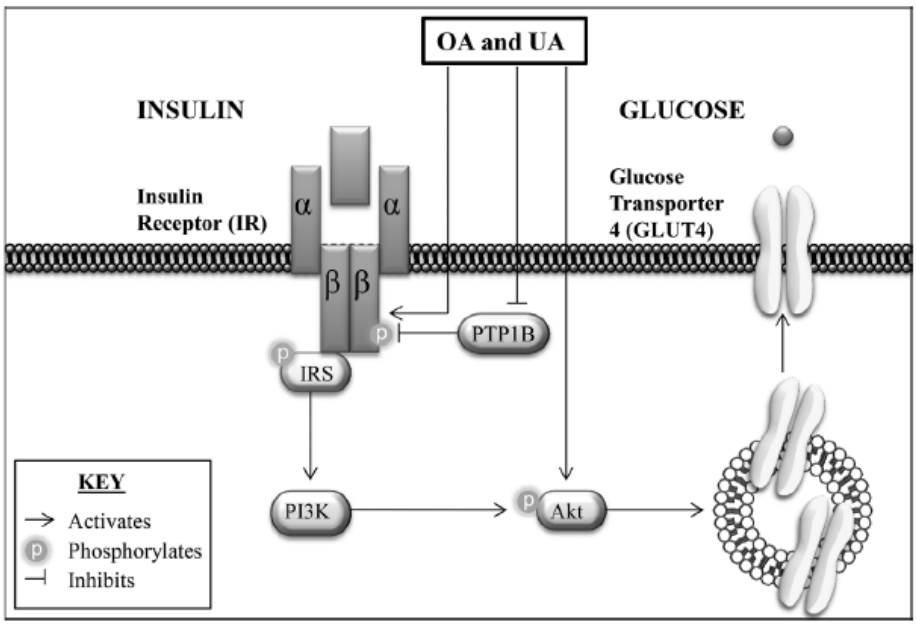

Figure 1. Ursolic acid (UA) and oleanolic acid (OA) effects on PTP1B inhibition of PI3K/Akt insulin signaling and glucose uptake in the target cell. Insulin binds to the insulin receptor (IR) at the α subunits resulting in a conformational change. Tyrosine residues on the β subunits phosphorylate resulting in downstream insulin signaling. PTP1B inhibits insulin signaling. Insulin receptor substrate (IRS) proteins are activated resulting in subsequent activation of phosphoinositide 3 kinase (PI3K) and Protein kinase B (Akt). In peripheral tissues including cardiac muscle, skeletal muscle, and adipose tissue, Akt activates the translocation of glucose transporter 4 (GLUT4), which is sequestered in vesicles before activation, into the plasma membrane. GLUT4 facilitates glucose uptake into the cell. Oleanolic acid (OA) and ursolic acid (UA) treatment inhibits PTP1B by directly binding to site B. This causes improved insulin signal transduction.

than their natural structures by 22 and tenfold in inhibition of PTP1B activity, respectively [25, 33]. PTP1B can inhibit the PI3K/Akt signaling pathway to induce insulin resistance by inhibiton of the translocation of GLUT4 to the plasma membrane [37] and causes disinhibition of FOXO1, thereby promoting reduction of glucose reuptake and gluconeogenesis. Therefore, the direct inhibition of PTP1B by OA, UA, and their derivatives enables signal transduction of insulin and thus improves insulin sensitivity.

In addition to insulin sensitization, inhibition of PTP1B also has the potential to promote weight loss, which is a benefit since obesity largely contributes to the type 2 diabetic pathology. Oral administration of OA, UA, and their derivatives decreased body weight gain in high-fat diet induced obese rodents [6–9, 15, 23]. PTP1B-deficient mice were resistant to weight gain and remained insulin-sensitive when subjected to a high-fat diet, while the amount of food consumed was not different [38]. The increased insulin sensitivity of PTP1B knockout mice cannot explain the reduced weight gain on a high-fat diet [38]. Several in vitro studies demonstrated that PTP1B is a negative regulator of the leptin-JAK2-STAT3 signaling pathway. The development of small molecule competitive inhibitors of PTP1B with modifying the UA and OA might provide novel therapeutic agents for obesity and type 2 diabetes.

5 OA and UA inhibit Keap1 causing Nrf2 activation and subsequent reduced oxidative stress and tissue damage

In type 2 diabetes, hyperglycemia promotes an increase in free radicals and decrease in anti-oxidants causing increased lipid peroxidation. Free radicals such as reactive oxygen species (ROS) can be detrimental since they can diffuse into cells causing damage to the mitochondrial enzymes and DNA, which subsequently leads to cellular dysfunction [9]. ROS are generated by oxidative stress such as the conversion of sorbitol to fructose in the polyl pathway [35]. In particular, ROS have been found to play a role in kidney fibrosis [39, 40]. A study has shown that damaged tubular cells in kidneys exacerbate ROS leading to apoptosis following unilateral ureteral obstruction [41].

One of the complications of type 2 diabetes is hepatocellular enzyme leakage, indicated by an increase in plasma enzyme activity of aspartate aminotransferase and alkaline phosphatase, which eventually results in severe liver damage [10]. A hepatoprotective effect of OA has been observed in diabetic mice through a reduction in the activity of alkaline phosphatase and aspartate aminotransferase, suggesting reduced hepatoxicity [10]. In animal studies, OA and UA treatment decreased liver damage induced by oxidative stress inducing chemicals such as carbon tetrachloride [42]. OA and UA also increased the activities of the anti-oxidant enzymes superoxide dismutase and glutathione peroxidase [10, 42, 43]. Increased levels of these anti-oxidant enzymes reduced the levels of free radicals lipid peroxidation [10]. The anti-oxidative effects of these compounds appear to be beneficial for the treatment and prevention of type 2 diabetes and associated complications, including oxidative stress induced liver damage.

Excess glucose in the blood promotes renal and hepatic tissue damage, and the polyl pathway is a major contributor to this damage. The function of this pathway is to metabolize unused glucose and it is activated during hyperglycemic states. The key enzymes in this pathway are aldose reductase (AR) and sorbitol dehydrogenase, which facilitate the production of sorbitol and fructose. The elevated sorbitol and fructose levels that occur due to the polyl pathway results in an increase in advanced glycation end product (AGE) formation and glycative injury [44, 46]. AGEs such as glycated

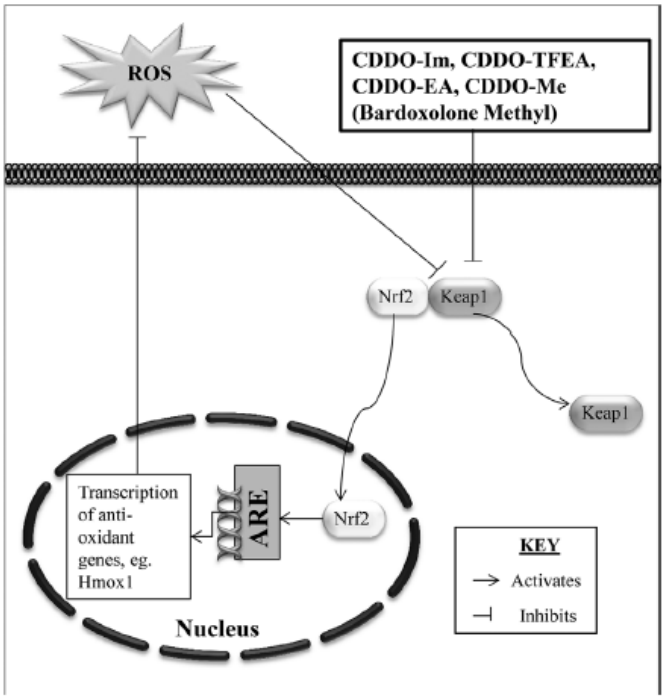

Figure 2. The effects of oleanolic acid (OA) CDDO derivatives on nuclear factor like 2 (Nrf2) activation. Oxidative stress such as reactive oxygen species (ROS) reduces the inhibitory activity of Kelch-like ECH-associated protein 1 (Keap1) on Nrf2. Nrf2 disassociates from Keap1 resulting in subsequent translocation of Nrf2 into the nucleus. Nrf2 binds to the anti-oxidant response element (ARE) to promote the transcription of a number of anti-oxidant genes such as Hmox1. Anti-oxidant enzymes transcribed by Nrf2 can inhibit ROS. The OA CDDO derivatives, CDDO-Im, CDDO-TFEA, CDDO-EA, and CDDO-Me (bardoxolone methyl) activate Nrf2 by reducing Keap1 inhibition of Nrf2.

hemoglobin ($HbAl_c$), N^{ε}-(carboxymethyl) lysine, and glycated albumin are thought to be involved in the development of diabetic nephropathy, with N^{ε}-(carboxymethyl) lysine and glycated albumin shown to contribute to its progression [47–50]. OA and UA administration in diabetic mice has been found to reduce the renal and liver activity of AR and sorbitol dehydrogenase and mRNA expression of AR causing suppression of the polyl pathway via decreased sorbitol and fructose production and AGE formation [13, 35]. OA treatment can also upregulate mRNA expression of glyoxalase I, an enzyme that metabolizes the AGE precursor methylglyoxal [13, 51]. The suppression of these molecules integral to the polyl pathway and inhibition of AGEs, including precursors, by OA and UA ameliorates liver and kidney injury [35]. This may hinder the progression of type 2 diabetes related complications of the liver and kidneys, including diabetic nephropathy, chronic kidney disease (CKD), and nonalcoholic fatty liver disease.

Nuclear factor like 2 (Nrf2) promotes the transcription of many anti-oxidant genes [52–54], and its intracellular interactions are summarized in Fig. 2. Nrf2 is usually bound to its inhibitor kelch-like ECH-associated protein 1 (Keap1) in the cytoplasm. An increase in oxidative or electrophillic stress inducing agents such as ROS causes Keap1 to lose its ability to inhibit Nrf2 which results in the disassociation of Nrf2 from Keap1 [55]. Nrf2 can then translocate into the nucleus where it binds to the anti-oxidant response element to stimulate the transcription of anti-oxidant genes [56–58]. The activation of Nrf2 and concurrent inactivation of Keap1 results in a reduction of oxidative stress and inflammation in a variety of tissues including the kidneys, liver, and retina. Nrf2 activation results in a reduction of blood urea nitrogen levels and the amelioration of glomerular and tubular injury in the kidneys [59]. In the livers of knockout and knockdown Keap1 mice, Nrf2 activation causes reduced hepatic inflammatory genes including IL-1β, IL-6, and TNF-α [60]. Following induced retinal ischemia reperfusion (IR), Nrf2 knockout mice have been found to have increased inflammatory cells, increased inducible nitric oxide synthase and oxidative stress compared to wild-type mice [57]. Nrf2 up-regulates the anti-apoptotic protein B-cell lymphoma (Bcl-2) and prevents cellular apoptosis [61]. In addition, Keap1 has been shown to promote a proinflammatory response through binding with the p65 subunit of nuclear factor kappa B (NF-κB) in HepG2 and HEK293 cells [62]. This suggests that Nrf2 activation influences both inflammation and oxidative stress. OA, UA, and derivatives have been found to have anti-inflammatory and anti-oxidative effects, which may be credited to Nrf2 activation. Briefly, the mechanisms behind this effect include inhibition of pro-inflammatory signaling and increasing the transcription of anti-oxidants; both of which are associated with Nrf2 activation. Therefore, further studies are warranted to determine if this effect is due to activation of Nrf2, which may be the optimum drug target for decreasing inflammation and oxidative stress.

OA and a number of synthetic derivatives of OA including CDDO-Me, CDDO-TFEA, CDDO-Im, and CDDO-Ea have been found to activate Nrf2 signaling [15, 72, 74]. OA has been found to increase Nrf2 activation and heme oxygenase 1 (Hmox1) expression causing a reduction in fibrosis and apoptosis in mice with unilateral ureteral obstruction [41]. CDDO-Me and CDDO-TFEA attenuate retinal damage, such as in diabetic retinopathy, via Nrf2 activation and subsequent transcription of several anti-oxidant genes including Hmox1, NADPH dehydrogenase quinone 1, and glutamate cysteine ligase catalytic subunit [57, 63]. The treatment of retinal IR induced mice with bardoxolone methyl increased retinal superoxide levels and reduced capillary degeneration by 60%. In addition to decreasing retinal damage, CDDO-Im has been found to induce the phosphorylation of Akt in retinal epithelial cells. On inhibition of the PI3K/Akt pathway, CDDO-Im treatment had no effect in inducing Hmox1 transcription, reiterating the relationship between Akt activation and Hmox1 expression [63]. This demonstrates a link between Nrf2 and Akt signaling pathways, and supports the previously described effect of these compounds on insulin signaling. This also suggests that the anti-oxidative and anti-inflammatory effects of Nrf2 activation may also be influenced by the activity of the PI3K/Akt pathway, perhaps through

inhibition of PTP1B. Therefore, Nrf2 activation by OA derivatives appears to be a promising target for reducing oxidative stress in type 2 diabetes and associated complications, such as diabetic retinopathy.

5.1 Clinical application of OA and an OA derivative: Bardoxolone methyl

OA is currently used as a dietary supplement in traditional Chinese medicine for treating liver injuries, inflammatory diseases, various types of cancers and diabetes [10, 64–67]. However, investigation of highly potent OA derivatives, including the OA CDDO derivative, bardoxolone methyl is still currently undergoing several human clinical trials to test its potential future use in a clinical setting. Bardoxolone methyl has successfully completed phase I and II of human clinical trials for treating CKD in type 2 diabetics, and phase I clinical trials for the treatment of leukaemia and solid tumors [68], indicating its potential in treating multiple diseases. The phase IIb human clinical trial study in 227 patients with type 2 diabetes and CKD showed that bardoxolone methyl improved kidney function with minimal side effects and no sign of hepatic injury [5]. The therapeutic effects of bardoxolone methyl were through upregulation of Nrf2 and Hmox1 expression in various regions of the kidneys [59]. Since bardoxolone methyl has successfully completed phase II of human clinical trials with positive benefits in patients with CKD and type 2 diabetes, this compound has potential clinical applications in the treatment of kidney disease in type 2 diabetes. The ability of bardoxolone methyl to activate Nrf2 may reduce oxidative stress and inflammation in other tissues such as the liver and retina, thereby ameliorating tissue damage in individuals with type 2 diabetes and prevent the development of associated microvascular and macrovascular complications. Further scientific investigation of the effect of bardoxolone methyl is needed in the future to determine if this drug has a similar effect in promoting Nrf2 activation in other tissues and organs and whether another molecule, such as PTP1B is responsible for its therapeutic effects.

Despite the number of benefits of OA and the OA derivative bardoxolone methyl, caution for specific populations should be taken when applied to patients with severe chronic kidney, hepatic, and/or heart diseases. In a clinical setting, the dose of OA can be as high as 80 mg three times per day for months in humans [69]. However, caution must be taken since it has been reported that OA can cause hepatotoxicity in long term use or if the dose is too high (>500 μmol/kg per day) in mice [69]. Phase III of human clinical trials testing bardoxolone methyl in patients with end stage CKD (stage 4 and up) was terminated due to a higher incidence of cardiovascular events compared to the placebo group [70]. Therefore, future human clinical trials using bardoxolone methyl should monitor blood pressure and heart function of participants, and overall caution should be taken in patients with a higher risk of cardiovascular events.

Recruitment for a human clinical trial in patients with pulmonary arterial hypertension (NCT02036970) is currently being undertaken in order to determine the efficacy and safety of bardoxolone methyl in this population, which is proposed to be completed by June 2015. This will aim to address safety issues in the phase III human clinical trials in advanced CKD patients.

6 OA and UA inhibit NF-κB and inflammatory cytokines resulting in reduced inflammation

Overnutrition leads to an accentuated proinflammatory state in several tissues including adipose tissue, liver, skeletal muscle, pancreas, and the hypothalamic region of the brain [71]. Inflammation of these tissues contributes to hyperglycemia, insulin resistance, and type 2 diabetes [71]. On a molecular level, proinflammatory signaling is mediated by NF-κB activation. The proinflammatory NF-κB signaling pathway in the target cell is summarized in Fig. 3. Briefly, NF-κB remains inactive when bound to and inhibited by nuclear factor kappa B inhibitor alpha (IκB) in the cytoplasmic region of the cell. NF-κB is activated by phosphorylated IκB kinase (IKK), which stimulates its translocation into the nucleus. Once NF-κB is in the nucleus, it regulates the expression of a variety of molecules such as the cytokine tumor necrosis factor alpha (TNF-α). TNF-α secreted due to the activation of the NF-κB can also increase the production of ROS, contributing to the development and progression of comorbidities associated with type 2 diabetes such as cardiovascular disease [72]. This proinflammatory signaling pathway is a positive feedback loop since TNF-α can bind to TNF receptor resulting in the phosphorylation and activation of IKK then subsequent NF-κB interaction. NF-κB activation can also promote the expression of the negative regulators of insulin signaling, PTP1B and suppressor of cytokine signaling 3 (SOCS3), thereby reducing insulin sensitivity and subsequent glucose regulation [61–64].

OA and UA have been found to reduce proinflammation by inhibiting proinflammatory signaling molecules and cytokines [73–75]. OA reduced NF-κB signaling by inhibiting LPS-induced phosphorylation of IkB, and subsequently the expression of the cytokines TNF-α and IL-1 [34, 76]. UA administration in mice fed a HFD also inhibited signaling through the NF-κB pathway [77]. The OA derivative, CDDO-Me has been found to directly influence proinflammatory signaling in Human U-937 myeloud leukemia cells by inhibiting IKK that causes blocking of the NF-κB pathway [78]. This OA derivative has also been found to suppress LPS induced inflammation in normal human PBMC cells by reducing the expression of the cytokines IL-6 and TNF-α [79]. However, a very high concentration of CDDO-Me was required to suppress NF-κB in macrophages, suggesting that NF-κB

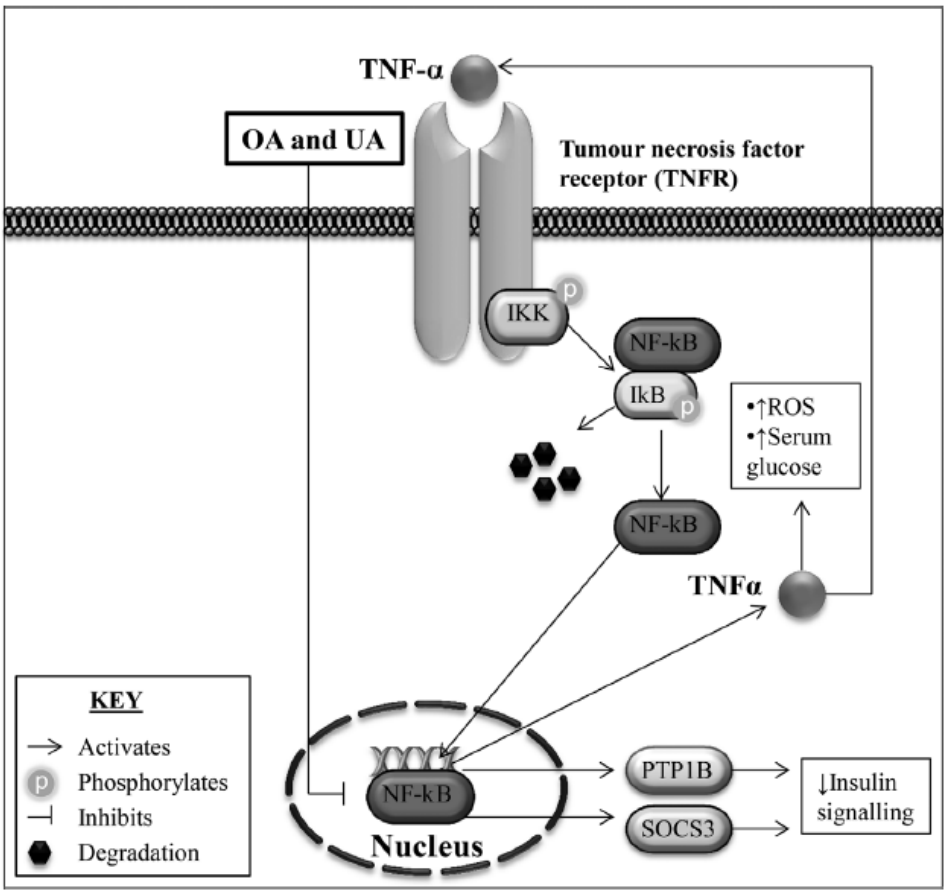

Figure 3. Ursolic acid (UA) and oleanolic acid (OA) effects on proinflammatory signaling in the target cell. Tumor necrosis factor alpha (TNF-α) binds to the TNF receptor. IκB kinase (IKK) is phosphorylated causing nuclear factor kappa B inhibitor alpha (IκB) phosphorylation. Disassociation of IκB from nuclear factor kappa B (NF-κB) and subsequent degradation. NF-κB is translocated into the nucleus where it functions to activate the transcription of a variety of molecules. NF-kB regulates the expression of TNF-α, protein tyrosine phosphatase 1B (PTP1B) and suppressor of cytokine signaling 3 (SOCS3), which are negative regulators of insulin signaling. TNF-α can then bind to the TNF receptor causing a detrimental feedback loop. UA has been found to inhibit NF-κB activation in mice, while OA has been found to reduce NF-κB translocation into the nucleus by inhibiting IκB phosphorylation.

signaling is not the only target by this compound and that its effects may occur through another pathway, possibly through Nrf2 [78, 80].

7 Nrf2, NF-κB, and PI3K/Akt signaling: Molecular pathways inextricably linked that contribute to the therapeutic effects of OA, UA, and derivatives

It has been found that Nrf2, NF-κB, and PI3K/Akt signaling pathways cross-talk. Several studies have demonstrated that an increase in PI3K/Akt activity has been linked to Nrf2 activation [81–85]. For example, recently, it was found that 3,4-dihydroxybenzalacetone administration enhanced Nrf2 activation, which was abolished with the treatment of a PI3K or Akt inhibitor, suggesting a role of the PI3K/Akt pathway in Nrf2 activation [85]. Furthermore, Nrf2 has been found to be activated as a result of NF-κB-induced inflammation and ROS production as a defensive response [86, 87]. The ability of OA, UA, and/or its derivatives to influence the activity of Nrf2, NF-κB, and the PI3k/Akt signaling suggests potential targets of these compounds in these molecular signaling pathways. Further studies are required to elucidate the exact mechanisms linking Nrf2, NF-κB, and PI3K/Akt pathways to induce the therapeutic benefits of OA, UA, and derivatives in type 2 diabetes and associated complications.

7.1 Conclusions, commentaries, and future directions

In summary, OA and its isomer, UA target signaling molecules that increase insulin signal transduction, and reduce inflammatory and oxidative stress signaling. OA and UA's promotion of insulin signaling has been demonstrated to occur through enhancement of IR-β subunit phosphorylation, upregulated Akt, and increased glucose uptake via GLUT4. In addition to improved Akt signaling, OA and UA also reduce glucose production by targeting FOXO1 and G6P. OA and UA directly inhibit the negative regulator of insulin signaling, PTP1B. In addition, OA and UA reduce inflammation through reduction of NF-κB signaling and inhibition of cytokines such as IL-6 and TNF-α, and increase antioxidant production via promotion of Nrf2 signaling.

Furthermore, modification of these triterpenes such as at C-28 leads to a higher potency in their interactions, such as in the anti-inflammatory and anti-oxidative properties of bardoxolone methyl. OA CDDO derivatives including bardoxolone methyl, have been found to reduce oxidative stress through activating Nrf2 signaling to stimulate antioxidant production, and reduce inflammation by reducing proinflammatory cytokine expression and NF-κB signaling. The potential of OA, UA, and their derivatives for clinical applications has been highlighted by bardoxolone methyl, which has been effective in phase II of human clinical trials for treating CKD

in patients with type 2 diabetes where activation of Nrf2 reduced tissue damage. However, caution should be taken in higher doses and particular populations such as patients with advanced stages of CKD. With further research and human clinical trials, the possibility of using OA, UA, and their derivatives for the treatment and prevention of type 2 diabetes and their complications appears promising.

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The authors have declared no conflict of Interest.

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Appendix 1.2 The Endothelin Pathway: A Protective or Detrimental Target of Bardoxolone Methyl on Cardiac Function in Patients with Advanced Chronic Kidney Disease?

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A1.2.1 Author Contributions

D.Camer reviewed the literature and wrote the first draft of the manuscript, which all authors reviewed and approved for publication.

A1.2.2 Collaborator Statement

I hereby declare that the statement in section A1.2.1 pertaining to the contributions of D.Camer is correct.

Prof Xu-Feng Huang

Perspective

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The Endothelin Pathway: A Protective or Detrimental Target of Bardoxolone Methyl on Cardiac Function in Patients with Advanced Chronic Kidney Disease?

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Key Words
Endothelin · Kidney · Renal · Heart · Bardoxolone methyl

Abstract
Bardoxolone methyl has been reported to cause detrimental cardiovascular events in the terminated BEACON Phase III human clinical trial via modulation of the renal endothelin pathway. However, the effects of bardoxolone methyl administration on the endothelin pathway in the heart are unknown. Our purpose in this perspective is to highlight the distinctive opposing roles of the renal and heart endothelin pathway in cardiac function. Furthermore, we address the need for further investigation in order to determine if bardoxolone methyl has a protective role in cardiac function through the suppression of the endothelin pathway in the heart.

The paradoxical nature of bardoxolone methyl (BM) has been widely noted in recent scientific literature [1]. There are many known anti-inflammatory and anti-oxidative properties of this drug that have shown promise in preclinical studies, including treating complications of diabetes such as retinopathy and nephropathy [2]. In addition, phases 1 and II of human clinical trials in patients with chronic kidney disease found that BM markedly improved kidney function [3, 4]. These positive results from the earlier phases led to the BEACON phase III human clinical trial in patients with type 2 diabetes and stage 4 chronic kidney disease [5]. However, this trial was terminated due to safety concerns centred on an increased incidence of cardiovascular events in BM-treated patients compared to the placebo group [6]. This has led to skepticism in the scientific community as to whether BM will ever be used in the future treatment of diseases such as chronic kidney disease.

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Table 1. Summary of endothelin system functions in the kidneys and vascular system

	ET-1	ET_A receptors	ET_B receptors
Kidneys	Regulates sodium and water homeostasis	Vasoconstrictor and sodium retention effects	Involved in water and sodium reabsorption and inhibiting vasopressin activity
Vascular system	Regulates vascular tone, cardiac hypertrophy and blood pressure	Vasoconstrictor (smooth muscle)	Vasoconstrictor (smooth muscle) and vasodilator (endothelial cells)

Mechanisms contributing to adverse cardiovascular events seen in patients treated with BM in the BEACON trial have since been addressed [7]. It was concluded that the modulation of the endothelin pathway provided an explanation of the mechanisms by which BM caused an increase in cardiovascular events in participants. It was also found that BM was able to suppress the endothelin pathway in the kidneys of chronic kidney disease (CKD)-induced rodents and healthy cynomolgus monkeys by reducing the protein expression of the ET_A receptor [7]. In healthy cynomolgus monkeys, BM did not affect the ET_B receptor expression [7]. However, in rodents induced with CKD, BM restored ET_B receptor levels to those observed in the control animals [7]. Thus, these results are extremely important in trying to explain the detrimental cardiovascular events that occurred in a number of patients taking BM in this human clinical trial. Despite this, there are limitations in arriving at this conclusion because only the endothelin pathway of kidney tissue was examined and not the cardiac tissue.

It is well established that renal endothelin 1 (ET-1) has several important functions including regulating sodium and water homeostasis, renal blood flow and acid base balance via activation of ET_A and ET_B receptors [8] (Table 1). Furthermore, if the endothelin system is suppressed, such as from being targeted by BM, this balance is disturbed causing fluid retention, which can lead to heart failure [8]. However, it is important to note that the endothelin pathway in the heart has a different function with regard to the kidneys. ET-1 in the cardiac muscle promotes cardiac hypertrophy and subsequent heart failure via activation of ET_A receptors [9] (table 1). Furthermore, both ET_A receptor and combined ET_A/ET_B receptor antagonism have been shown to lower blood pressure and reduce the infarct size in patients with congestive heart failure [9]. Thus, suppressing the endothelin pathway in the heart would be protective against adverse cardiovascular vascular events.

In our opinion, due to the differing functions of the endothelin pathway in the kidneys and heart, we feel that more evidence is required to conclude that BM treatment causes adverse cardiovascular events by suppressing the renal endothelin pathway. Future investigation of the effects of BM on cardiac tissue is required in order to determine if BM inhibits ET_A receptors in the heart causing its protection.

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Appendix 1.3 Comment on: Oleanolic acid co-administration alleviates ethanol-induced hepatic injury via Nrf-2 and ethanol-metabolizing modulation (sic) in rats

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A1.3.1 Author Contributions

D.Camer reviewed the literature and wrote the first draft of the manuscript, which all authors reviewed and approved for publication.

A1.3.2 Collaborator Statement

I hereby declare that the statement in section A1.3.1 pertaining to the contributions of D.Camer is correct.

Prof Xu-Feng Huang

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Letter to the Editor

Comment on: Oleanolic acid co-administration alleviates ethanol-induced hepatic injury via Nrf-2 and ethanol-metabolizing modulation (sic) in rats

To the Editor:

Alcohol induced hepatic oxidative stress and inflammation is known to cause liver injury. An increase in reactive oxidative species (ROS) from alcohol consumption leads to oxidative stress [1]. This can activate the inflammatory cytokines, IL-6 and TNF-α which promote liver injury. Both IL-6 and TNF-α are activated and transcribed by the inflammatory molecule, NFκB [2]. We read the interesting paper by Liu et al., entitled, "Oleanolic acid co-administration alleviates ethanol-induced hepatic injury via Nrf-2 and ethanol-metabolizing modulating in rats", published in your journal recently [3]. The authors demonstrated that oleanolic acid can reduce hepatic injury by elevating Nrf-2 related antioxidants, reduce inflammation, and increase ethanol metabolism. We believe that the mechanism of modulating these signalling pathways could be important for understanding the protective effects of oleanolic acid.

Firstly, the reduction of oxidative stress and inflammatory signalling pathways by oleanolic acid may contribute to decreased liver injury as shown by the authors. In this study, it has been shown that IL-6 and TNF-α elevation was attenuated by oleanolic acid administration, however, NFκB was not examined. Oleanolic acid has been found to reduce NFκB signaling by inhibiting LPS-induced phosphorylation of IkB, and subsequently the expression of the cytokines TNF-α and IL-1 [4]. Thus, a reduction in NFκB signalling by oleanolic acid may cause the reduced expression of the cytokines IL-6 and TNF-α found in this study. However, whether oleanolic acid directly targets NFκB in alcohol induced liver injury remains unknown. Therefore, whether oleanolic acid can directly inhibit NFκB leading to a subsequent reduction in activation and transcription of inflammatory cytokines may also be important in the attenuation of alcohol induced liver injury. Oleanolic acid has been shown to directly inhibit intracellular signalling molecules including PTP1B, a molecule that can be activated by NFκB [5]. This interaction occurs by oleanolic acid binding directly to site B of PTP1B, leading to its inhibition.

In addition, it has been found that Nrf2 and NFκB signalling pathways cross-talk [6]. Nrf-2 has been found to be activated as a result of NFκB induced inflammation and ROS production as a defensive response. Nrf-2 activation also causes reduced hepatic inflammatory genes including IL-6, and TNF-α. Therefore, it would be interesting to compare the effects of oleanolic acid on the activity of both Nrf-2 and NFκB signalling pathways in order to determine their role in alcohol induced liver injury.

In conclusion, the ability of oleanolic acid to influence the activity of Nrf-2 and NFκB signalling suggests potential targets of this compound in these molecular signalling pathways. Further studies are required to elucidate the exact mechanisms linking Nrf-2 and NFκB to induce the therapeutic benefits of oleanolic acid in alcoholic induced liver disease.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Transparency Document

The Transparency Document associated with this article can be found in the online version.

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Appendix 1.4 Is B-type Natriuretic Peptide a Risk Factor for Heart Failure in Patients Treated With Bardoxolone Methyl?

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A1.4.1 Author Contributions

D.Camer reviewed the literature and wrote the first draft of the manuscript, which all authors reviewed and approved for publication.

A1.4.2 Collaborator Statement

I hereby declare that the statement in section A1.4.1 pertaining to the contributions of D.Camer is correct.

Prof Xu-Feng Huang

Letters to the Editor

Is B-type Natriuretic Peptide a Risk Factor for Heart Failure in Patients Treated With Bardoxolone Methyl?

To the Editor:

We read with great interest the article entitled "Risk Factors for Heart Failure in Patients With Type 2 Diabetes Mellitus and Stage 4 Chronic Kidney Disease Treated With Bardoxolone Methyl" by Chin et al[1] in a recent issue of the *Journal of Cardiac Failure*. The authors reported that patients treated with bardoxolone methyl in the BEACON phase III human clinical trial with a baseline B-type natriuretic peptide (BNP) of ≥200 pg/mL or with earlier hospitalization had a 60% increased risk of heart failure compared with the placebo group. We think that this information is crucial to design successful human clinical trials investigating the potential benefits of bardoxolone methyl in the future. However, we have some additional comments regarding the outcome of the study.

First, in the article's Figs. 1, "Classification tree for heart failure events in BEACON," and 2, "Classification tree for fluid overload or heart failure events," the baseline BNP data in the classification trees is set to a different level for the bardoxolone methyl group (±183.pg/mL) than for the control group (±229.5 pg/mL). Furthermore, a study by Wang et al reported that BNP levels as low as 20 pg/mL can increase the risk of heart failure.[2] In addition, the conventional values for diagnosis of heart failure are usually between 80 and 100 pg/mL.[3,4] Therefore, an equal comparison between baseline BNP levels and both the bardoxolone methyl and the placebo groups in the BEACON clinical trial is necessary. A consideration of the risk of heart failure in patients treated with bardoxolone methyl compared with placebo with lower BNP levels would also be interesting to consider in future studies.

Second, there is controversy surrounding the usefulness of BNP as a biomarker for heart failure mechanisms, because levels vary widely in the general population based on factors including age, sex, and body mass index (BMI).[5,6] Specifically, elevated BNP levels can occur in conditions independently from heart failure, including old age, the use of hormone replacement therapy, and obesity.[7] In the Chin et al study, patients with type 2 diabetes and stage 4 chronic kidney disease were divided only into a bardoxolone methyl treatment or a placebo group and were not divided according to other parameters, such as age, sex, and BMI. Therefore, whether there were differences between these factors in the BEACON trial may also be interesting parameters to investigate to eliminate potential non−heart failure mechanisms that have the ability to elevate BNP levels.

In conclusion, despite the risk factors for heart failure identified by Chin et al, the benefits of bardoxolone methyl in earlier human clinical trials and preclinical animal studies cannot be ignored.[8] Future studies should focus on identifying molecular mechanisms in the heart and the cardiovascular network that explain potential increased risks of the development of heart failure in patients treated with bardoxolone methyl.[9] This will allow the development of more robust human clinical trials using bardoxolone methyl in the future.

Disclosures

The authors have nothing to disclose.

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Reply:

We thank Drs Camer and Huang for their interest in our work. We would like to clarify a few points regarding the B-type natriuretic peptide (BNP) thresholds that resulted from our classification and regression tree (CART) analysis for heart failure events in BEACON. The baseline BNP values of the bardoxolone methyl and placebo patients in BEACON have previously been reported[1] and were not statistically different across treatment groups. Additionally, the thresholds for baseline BNP (bardoxolone methyl: 183.5 pg/mL; placebo: 229.5 pg/mL) were not selected a priori. Rather, with the use of the methods described in the paper, the best classification tree was selected to minimize cost (ie, classification error) of patients within each treatment group who had heart failure events. Therefore, although we agree that other thresholds for BNP prediction have been previously reported to increase the risk of heart failure in other patient populations, the results from the present analyses indicate that bardoxolone methyl increased the risk of heart failure in the diabetic stage 4 chronic kidney disease (CKD) BEACON patients with BNP greater than ~200 pg/mL and with a history of heart failure hospitalizations. Notably, as mentioned, patients with lower BNP (ie, normal or near-normal BNP) and no history of heart failure did not experience an increased risk of heart failure or fluid overload adverse events with bardoxolone methyl treatment.

Second, several other baseline characteristics were evaluated as potential risk factors for heart failure in the CART analysis, including baseline albuminuria (urine albumin-to-creatinine ratio, serum creatinine, age, prescription of inhibitors of the renin-angiotensin-aldosterone system). None of these other factors was identified as a significant predictor of heart failure in BEACON. As stated, we selected CART as the method of choice because we did not want to limit the form of interaction terms to those easily defined by logistic or similar regression models. We performed a similar analysis with the use of logistic regression as another method for identifying risk factors, and included other characteristics such as age, baseline body mass index, and weight. This approach led to the same risk factors as identified by CART; however, diagnostics showed that the models were poor fits to the data, probably because they were unable to capture the nature of the interaction among the risk factors.

Additional analyses to identify potential mechanisms that contribute to the risk of heart failure in patients with bardoxolone methyl have been detailed elsewhere.[2,3] Notably, the clinical phenotype of the events that were adjudicated as heart failure was severe fluid overload requiring intravenous diuretics. Hospital records revealed that these events were associated with preserved cardiac and renal function. At baseline and before randomization, bardoxolone methyl–treated patients who experienced these events had BNP values on average >500 pg/mL, demonstrating that they were already fluid overloaded.

Collectively, these data suggest that bardoxolone methyl treatment may differentially affect systemic hemodynamic status according to the clinical condition of subjects, having no clinically detectable effect in healthy volunteers or early-stage CKD patients, but apparently promoting fluid retention in patients with more advanced renal dysfunction and the recognized risk factors associated with heart failure at baseline. Moreover, multiple findings suggest potential clinical benefit where risk of fluid retention is limited; data from BEACON showed improvement in multiple renal function parameters, reduction of muscle inflammation and injury markers, and improved metabolic parameters.[3] On the basis of these data and the results from the present work, bardoxolone methyl is currently being studied in the United States in patients with pulmonary arterial hypertension who do not have advanced renal dysfunction or elevated BNP (NCT 02036970). The drug is also being studied in Japan in diabetic stage 3 CKD patients who do not have the previously mentioned risk factors for fluid retention.

Disclosures

Both authors are employees of Reata Pharmaceuticals.

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