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Abstract

Scope: Energy deficit is a common characteristic of neurodegenerative disorders, including Alzheimer's disease. Adenosine monophosphate activated protein kinase (AMPK) is a key enzyme maintaining energy balance by regulating the cellular uptake of glucose, β -oxidation of fatty acids, and expression of glucose transporter 4. Since resveratrol has been shown to increase the activity of AMPK, we hypothesized that it might influence energy metabolism in a model neuron-like cell line, murine Neuro2a cells.

Methods and results: Resveratrol caused an elevation of adenosine triphosphate (ATP) and guanosine triphosphate (GTP) in a dose-dependent manner. The highest ATP and GTP levels achieved by treatment with resveratrol were 70.3 ± 8.2 nmol/mg protein (1.9-fold of control) and 27.2 ± 4.0 nmol/mg protein (1.7-fold of control), respectively, when cells were treated with 100 μ M resveratrol for 6 h. Interestingly, increases in the total sum of all adenine nucleotides were found upon addition of resveratrol. Despite these increases in ATP, GTP, and the total adenine nucleotide pool, resveratrol treatment led to a decrease in glucose consumption and lactate release, suggesting that resveratrol does not increase energy production (e.g. via AMPK kinase activation) but rather inhibits energy-consuming processes.

Conclusion: Resveratrol increases the levels of ATP and GTP, but without creating an additional glucose demand.

Keywords

polyphenols, energy levels, neuronal cell line

Disciplines

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Pro-energetic effect of resveratrol in the murine neuronal cell line Neuro2a

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Abbreviations

A β , Beta-amyloid; **AD**, Alzheimer's disease; **ADP**, Adenosine diphosphate; **AMP**, Adenosine monophosphate; **AMPK**, AMP-activated protein kinase; **ATP**, Adenosine triphosphate; **CaMKK β** , Calcium/calmodulin-dependent protein kinase kinase- β ; **DMEM**, Dulbecco's Modified Eagles Medium; **EDTA**, Ethylenediamine tetraacetic acid; **FBS**, Fetal bovine serum; **GDP**, Guanosine diphosphate; **GTP**, Guanosine triphosphate; **HPLC**, High performance liquid chromatography; **LKB1**, Liver kinase B-1; **NAD⁺**, Nicotinamide adenine dinucleotide; **NADP⁺**, Nicotinamide adenine dinucleotide phosphate; **TBA**, Tetrabutylammonium hydrogen sulphate; **ULK1**, unc-51-like kinase-1; **WVL**, Wave length.

Abstract

Scope: Energy deficit is a common characteristic of neurodegenerative disorders, including Alzheimer's disease. AMP-activated protein kinase is a key enzyme maintaining energy balance by regulating the cellular uptake of glucose, β -oxidation of fatty acids and expression of glucose transporter 4. Since resveratrol has been shown to increase the activity of AMPK, we hypothesized that it might influence energy metabolism in a model neuron-like cell line, murine Neuro2a cells.

Methods and Results: Resveratrol caused a significant elevation in the levels of the energy-rich carriers ATP and GTP in a dose-dependent manner. The highest ATP and GTP levels achieved by treatment with resveratrol were 70.3 ± 8.2 nmol/mg protein (1.9 fold of control cells) and 27.2 ± 4.0 nmol/mg protein (1.7 fold of control cells), respectively, when cells were treated with 100 μ M resveratrol for 6 h. Interestingly, substantial increases in the total sum of all adenine nucleotides were found upon addition of resveratrol. Despite these increases in ATP, GTP and the total adenine nucleotide pool, resveratrol treatment led to a pronounced decrease in glucose consumption and lactate release, suggesting that resveratrol does not increase energy production (e.g. via AMPK kinase activation) but rather inhibits energy consuming processes.

Conclusions: Resveratrol increases the levels of free high-energy nucleotides, including ATP and GTP, but without creating an additional glucose demand.

Introduction

Alzheimer's disease (AD) is the most common form of dementia in the elderly [1], resulting in gradually impaired memory, learning and communication skills [2]. With the use of positron emission tomography, it has been shown that AD brains display evidence of a reduction in glucose metabolism even in the early stages of the disease [3, 4]. For example, findings by Sakamoto and colleagues [5] indicated that both early-onset and late-onset AD patients show marked hypo-metabolism in the bilaterally parieto-temporal regions of the brain, compared to the age-matched control subjects. Therefore, diminished brain energy metabolism is postulated as one of the contributing factors for neuronal dysfunction and cell death and the resulting cognitive deficits in AD [6, 7].

Adenosine triphosphate (ATP), a multifunctional high-energy molecule is present in all metabolically active cells [8]. Neuronal cells in particular have a high metabolic activity and are highly dependent on energy production via glucose metabolism for active transport and maintenance of ion homeostasis [9, 10]. Specifically, high levels of ATP are required to recover Na^+ and K^+ distribution across the plasma membrane, providing an electrochemical gradient necessary for maintenance of the resting membrane potential and control of osmotic balance [11], as well as for the formation and release of neurotransmitters and synaptic function [12, 13].

A progressive reduction in ATP levels relating to the degree of neurodegeneration in the AD brain has been identified in sporadic AD patients [14, 15]. According to the study of Hoyer [14], a decrease in the cerebral ATP generation rate was found to range from at least 7% in incipient early-onset AD to approximately 19% in incipient late-onset AD, while more than 50% ATP generation rate was reduced in stable advanced AD. In addition, Sims and

colleagues [15] also have shown that ATP levels in the neocortical tissue of AD brains were significantly lower than in healthy controls.

AMP-activated protein kinase (AMPK), one of the key enzymes regulating cellular energy homeostasis, is a promising target for the treatment of metabolic disorders [16, 17]. AMPK is a crucial cellular energy sensor. Once activated by falling energy status, it promotes ATP production by increasing the activity or expression of proteins involved in catabolism while conserving ATP by switching off biosynthetic pathways. Activated AMPK suppresses ATP consumption pathways, including cell growth, protein synthesis, fatty acid synthesis and ribosome biosynthesis, whereas stimulating ATP generation pathways such as glucose uptake, glycolytic flux, fatty acid oxidation and mitochondrial biogenesis [18, 19]. AMPK activation plays a protective role in metabolic stress conditions, such as ischemia, hypoxia, oxidative stress and starvation [18, 20, 21] and is modulated by exercise and dietary hormones, including leptin and adiponectin [22, 23]. AMPK activation facilitates dietary food intake regulated by the hypothalamus [22], glucose uptake and fatty acid oxidation in heart and skeletal muscle [24, 25], whilst it suppresses fatty acid and cholesterol synthesis in the liver [26].

Resveratrol (*trans*-3,4',5-trihydroxystilbene), a naturally occurring polyphenol, is present in the skin of red grapes, some fruits and herbs such as *Polygonum cuspidatum* at high levels and possesses strong antioxidant properties [27]. Resveratrol has been found to activate AMPK in neuroblastoma cells and primary neurons *in vitro* as well as in the brain by activating calcium/CaMKK β /AMPK signaling pathways [28, 29].

In this study, we have investigated the effect of resveratrol on neuronal energy levels, and therefore initially measured its effects on levels of ATP and GTP. Further, the effect of resveratrol on glucose consumption and lactate release of Neuro2a cells was investigated in order to determine whether the observed pro-energetic effect of resveratrol was caused by an increase in aerobic glucose consumption. Together our data identifies a new role for resveratrol, increasing the total adenine nucleotide content and inhibiting energy consuming processes in Neuro2a cells.

Materials and Methods

Materials

The murine neuroblastoma Neuro2a cell line was obtained from ATCC (South Granville, Australia). All cell culture materials were from Invitrogen (Mulgrave, Australia). Coomassie Blue Brilliant G-250 powder for Bradford assay was from Amresco (Astral, Sydney, Australia). Chromatography grade acetonitrile was from Merck (Kilsyth, Australia). HPLC buffers were prepared with ultra-pure water filtered through Milli-Q system (Millipore, North Ryde, Australia). *Trans*-Resveratrol was provided by Nutrafur (Alcantarilla, Spain). All other reagents and chemicals were from Sigma-Aldrich (Castle Hill, Australia).

Cell maintenance

Neuro2a cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 25 mM glucose, supplemented with 5% heat-inactivated foetal bovine serum (FBS), 2 mM L-glutamine (Glutamax), antibiotics (200 U/mL penicillin, 200 µg/mL streptomycin) and fungicide (2.6 µg/mL Fungizone). The cell line was maintained in 5% CO₂ at 37°C and passaged every 3-4 days in 75 or 175 cm² tissue culture flasks. For experiments, Neuro2a were harvested by cell scraper, counted with Trypan blue and seeded in 12-well plates at a density of 0.55x10⁶ cells/well in 2 mL DMEM containing 2% FBS and incubated for 24 hours before treatment with resveratrol.

Treatment of Neuro2a cells with resveratrol

A stock solution of resveratrol (100 mM) was prepared immediately before use by dissolving in absolute ethanol. Neuro2a cells were incubated with medium containing resveratrol at a range of concentrations (12.5, 25, 50, 75, 100 µM) and in a final concentration of 0.1% ethanol. Control cells were treated with medium containing 0.1% ethanol. Intracellular energy

levels were measured after 1, 6, 12, 24 and 48 h incubation with resveratrol. At each time point, the cells were washed three times with phosphate buffered saline, extracted with ice-cold HClO_4 0.5M and neutralized with ice-cold K_2CO_3 2M to pH 7. The supernatants were stored at -80°C prior to analysis by HPLC. The cell pellets were dissolved in NaOH 1M, then protein content was determined by the Bradford assay. At the time points of 24 h and 48 h, 1 mL medium from each well was collected and centrifuged at 1000 $\times g$ for 3 min and the supernatant was frozen at -80°C prior to glucose and lactate assay.

Measurement of intracellular nucleotide levels

50 μL supernatant of each sample was injected into the stationary phase and analyzed by gradient ion-pair reversed-phase HPLC [30]. Briefly, the mobile phase was composed of buffer A (100 mM KH_2PO_4 , 8 mM TBA, pH 6.0) and buffer B (100 mM KH_2PO_4 , 8 mM TBA, 30% acetonitrile (v/v), pH 6.0) at 1.5 mL/min flow rate. The stationary phase system was a Supelcosil-LC-18-T column (150x4.6 mm, 3 μm) and a Supelguard LC-18-T Guard Column (Supelco, Castle Hill, Australia). Nucleotides were detected at the wavelength of 254 nm with a photodiode array PDA ICS-3000 detector (Dionex, Lane Cove, Australia). Stock solutions of 5 nucleotides of interest including ATP, ADP, AMP, GTP and GDP were prepared in KH_2PO_4 0.1M pH 7.0 and then mixed together to give the top concentration of 100 μM for each nucleotide. This mixture was then diluted in KH_2PO_4 0.1M, pH 7.0 by a factor of 2 to form standard solutions of decreasing concentration from 100 to 1 μM .

Determination of glucose levels in media

The medium samples were defrosted and diluted 1:8 with distilled water. Glucose concentration in each sample was determined by an enzymatic reaction previously described [31] with a slight modification. 20 μ L of each diluted sample was added to each well of a flat bottom 96-well plate in duplicate, followed by 180 μ L of a reaction mixture composed of 1 U/mL hexokinase/glucose-6-phosphate dehydrogenase, 2 mM NADP⁺, 6 mM ATP, 3 mM MgSO₄ in 0.3 M triethanolamine buffer, pH 7.6. After being incubated for 2 hours at room temperature, absorbance at 340 nm was measured using a microplate reader (BMG Labtech, Mornington, Australia). A standard curve of glucose in the range from 0 to 3 mM was fit with a linear regression.

Determination of lactate levels in media

The medium samples were defrosted and diluted 1:8 with distilled water. Lactate concentration in each sample was determined by an enzymatic reaction previously described [32] with a slight modification. 20 μ L of each diluted sample was added to each well of a flat bottom 96-well plate in duplicate, followed by 180 μ L of a reaction mixture consisting of 50 U/mL lactate dehydrogenase and 10 mM NAD⁺ in a 0.2 M hydrazine – 0.2 M glycine buffer containing 5 mM EDTA, pH 9.5. After being incubated for 1 hour at 37 °C and 20 minutes subsequently at room temperature, absorbance at 340 nm was measured with a microplate reader (BMG Labtech, Mornington, Australia). A standard curve of lactate in the range from 0 to 3 mM was fit with a linear regression.

Determination of total protein

Total protein content was assessed by Bradford assay [33]. Bradford solution was made by dissolving 100 mg Coomassie Blue Brilliant G-250 in 50 mL ethanol 100% and 100 mL

phosphoric acid 85%, then diluted to 1L with double distilled water and filtered through Whatman No.1 filter paper. To evaluate total protein amount of cell lysates, 10 μ L of each sample was added to each well of 96-well flat bottom plate in duplicate, followed by 200 μ L Bradford solution. Absorbance was measured at 595 nm using a microplate reader (Bio-Rad, Gladesville, Australia). A standard curve in the linear range was constructed by dissolving bovine serum albumin in a range of 0.05 to 0.5 mg/mL in NaOH 1M.

Neutral Red uptake assay for cell viability assessment

To determine cell viability, the incubation medium was removed from wells and replaced with Neutral Red medium (50 μ g/mL). After being incubated for 2 hours at 37°C and 5% CO₂, Neutral Red medium was removed from the cells and the cells were washed once with phosphate buffered saline and extracted with destaining solution (50% absolute ethanol, 49% distilled water and 1% glacial acetic acid). The plates were shaken on a micro-plate shaker for at least 10 minutes until the Neutral Red was completely removed from cells. The absorbance was measured at 540 nm with a microplate reader (Bio-Rad, Gladesville, Australia). Values were expressed as a percentage of untreated control cells after subtracting mean blank readings in the absence of cells.

Statistics

Data were analysed and displayed in figures using GraphPad Prism 5. Values presented are the mean of 3 independent experiments in duplicate and error bars denote standard error of the mean (SEM). Significant differences were assessed by Student's t-test for comparison between 2 groups or one-way ANOVA with Dunnett's multiple comparison test for comparison of 3 or more groups, and significance is shown as * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

Results

Effect of resveratrol on proliferation of the Neuro2a cell line

The cell number and the total protein content at each timepoint and at each resveratrol concentration was determined to allow us to normalize the nucleotide phosphate concentrations in the later experiments. Therefore, Neuro2a cells were incubated with different concentrations of resveratrol (ranging from 12.5 μ M to 100 μ M) for 24 and 48 h. The number of cells at each time point was determined using a phagocytosis based cell viability assay (Neutral Red) and compared to the number of cells present at 0h. The number of untreated Neuro2a cells increased to 151% (at 24 h) and 166% (at 48 h). With increasing resveratrol concentrations, proliferation decreased in a dose dependent manner (Fig. 1A). Total protein content followed a similar dose-response curve (Fig. 1B). At 100 μ M resveratrol, cell numbers at 24 and 48 h fell below the cell number at the starting point (Fig. 1A,B). For this reason, all following experiments, used a dose range of 12.5 μ M to 100 μ M resveratrol, whereby 12.5 μ M was considered as the “highest non-toxic” concentration, 50 μ M as an “antiproliferative” and 100 μ M as a “cytotoxic” concentration.

Resveratrol significantly increases the levels of the high-energy nucleotides ATP and GTP in a dose-dependent manner

ATP and GTP are the most important energy carriers in living cells. To investigate the effects of resveratrol on the concentrations of these nucleotides, Neuro2a cells were treated with resveratrol at concentrations ranging from 0 to 100 μ M. ATP and GTP levels were determined after 1, 6, 12, 24 and 48 h of incubation.

In the untreated control cells, the levels of ATP and GTP decreased during the incubation period, from approximately 42.0 nmol ATP and 20.3 nmol GTP/mg protein (1 h) to 26.0 nmol ATP and 13.2 nmol GTP/mg protein (24 h) and drastically deteriorated at 48 h, where ATP and GTP levels were approximately 11.4 and 7.0 nmol/mg protein, respectively (Fig. 4A, 4B).

The addition of resveratrol led to a significant elevation in intracellular ATP levels, compared to untreated controls, in a dose-dependent manner (Fig. 2, 4A). This effect could be observed after 1 h of treatment and these increases lasted up to 48 h (Fig. 4A). The highest levels of ATP observed were 70.3 ± 8.2 nmol/mg protein (1.9 fold higher than in non-treated control cells), measured at the 6 h time-point and at a concentration of 100 μ M resveratrol (Fig. 2, 4A).

Resveratrol did also increase GTP levels at all time points up to 48 h (Fig. 4B). The highest GTP level observed after treatment with resveratrol was 27.2 ± 4.0 nmol/mg protein (equal to 1.7 fold higher than non-treated control cells), which was also reached at 6 h following treatment with 100 μ M resveratrol (Fig. 2, 4B).

Resveratrol leads to an increase in the levels of AMP and GDP

ADP, AMP and GDP are low-energy nucleotides derived from the hydrolysis of one or two high energy phosphate bonds of ATP and GTP. We examined their levels in Neuro2a cells after addition of resveratrol to investigate the effects of resveratrol on energy metabolism. If activation of AMPK would be the major mode of action of resveratrol, one would expect an increase in energy charge, evident by a decrease in the concentration of these low energy nucleotides compared to ATP and GTP.

Surprisingly, resveratrol also caused a dose-dependent increase in ADP and AMP levels in Neuro2a cells as early as 1 h and up to 24 h after treatment. No significant changes in GDP levels could be observed between the non-treated control and resveratrol-treated cells except at 48 h (Fig. 3).

At 48 h (when nutrient levels in control cells appear to be depleted), AMP and GDP levels of the non-treated controls dramatically increased whilst ATP and GTP levels markedly declined (compare Fig. 3, 4 and 5). It is possible that some essential nutrients in the cell culture were exhausted due to proliferation and therefore cells could not sufficiently replenish ATP and GTP from AMP, ADP and GDP. Therefore, a large amount of ATP was hydrolyzed to the low-energy phosphate AMP (Fig. 5B), and GTP was hydrolysed to GDP (Fig. 5C) in untreated Neuro2a cells. Resveratrol treatment significantly lowered the levels of low-energy nucleotides AMP and GDP levels by more than 50% in Neuro2a cells after 48 h, compared to control values (Fig. 5B, 5C).

Resveratrol significantly increases the total pool of adenine nucleotides

We examined the effect of resveratrol on the total adenine nucleotide content in Neuro2a cells. The results showed that the sum of adenine nucleotides in untreated control cells also decreased over the 48 h incubation period consistent with the expected reduction in ATP levels and ranged from 49.3 ± 5.3 nmol/mg protein (1 h) to 25.1 ± 2.4 nmol/mg protein (48 h) (Table 1).

In cells treated with resveratrol, a substantial elevation in total adenine nucleotide levels of Neuro2a, compared to control cells was observed in a dose-dependent manner. This effect was apparent at all experimental time-points. The highest value of total adenine nucleotide content obtained after treatment with resveratrol was 81.4 ± 8.6 nmol/mg protein (equivalent to 1.8 fold of the control cells), at 6 h following treatment with 100 μ M resveratrol.

Resveratrol does not change the cellular energy charge potential (ECP) ratio during the stages of sufficient nutrition, but improves that ratio at the stages of starvation

As first proposed by Atkinson and Walton in 1967 [34], the energy charge potential (ECP) or adenylate energy charge (AEC) is an index used to measure the energy status of biological cells. ECP is calculated by the following equation: $ECP (\%) = (ATP + \frac{1}{2} ADP) \times 100 / (ATP + ADP + AMP)$. In theory, ECP can vary from 0% (all AMP) to 100% (all ATP); however, ECP is normally maintained in the range of 70% to 100% in healthy cells and tissues that are provided with sufficient nutrition and oxygenation [35, 36]. Thereupon, ECP can be seen as an indicator of “well-being” status of cells and organisms [37].

We assessed the effect of resveratrol on ECP during 48 h experiments. The results show that there were no statistical differences between ECP of untreated cells and cells treated with

resveratrol during the first 24 hours, with ECP remaining at high levels (> 80%) suggesting a balanced metabolic status in the cells (Fig. 6A). Previous experiments suggested that 48 h of resveratrol led to a marked increase in the low-energy nucleotide AMP and a substantial decrease in the high-energy carrier ATP (Fig. 3, 4, 5) since the nutrient source in the culture medium was consumed and exhausted over the time of incubation, resulting in a drop of ECP. Hence, we investigated whether resveratrol could reverse this phenomenon. At the 48 h time-point, ECP drastically reduced to under 70% in control cells, indicating energy depletion occurring (Fig. 6B). Resveratrol, however, suppressed this phenomenon as it elevated the ECP to over 80% at all concentrations of resveratrol treatment from 12.5 μ M to 100 μ M (Fig. 6B).

Resveratrol induces pronounced decreases in glucose consumption by Neuro2a cells

Because glucose is the major energy source for living cells to produce ATP and GTP [38], we investigated whether the mechanism behind these resveratrol-induced increases in high-energy phosphates was due to an increase in glucose uptake.

Glucose consumption or glucose uptake was calculated based on the decrease in glucose concentration in the media after 24 h or 48 h. The outcomes show that although resveratrol treatment resulted in a significant increase in ATP and GTP, this was not accompanied by higher glucose consumption (Table 2 and Table 3). During the first 24 hours, resveratrol at the concentrations of 12.5 μ M, 25 μ M, 50 μ M, 75 μ M and 100 μ M caused a reduction in glucose consumed by Neuro2a cells to 20%, 30%, 43%, 55% and 60%, respectively, whereas a reduction in cell viability was only 4%, 9%, 23%, 30% and 48%, respectively (Table 2). Similarly, resveratrol-induced decreases in glucose consumption by the Neuro2a cell

population were also observed following the 48 h treatment with resveratrol (Table 3). Therefore, the values of glucose consumption normalized to cell viability of Neuro2a cells were considerably lower than controls. These data therefore suggest that despite resveratrol-induced ATP and GTP increases, resveratrol decreased glucose consumption by Neuro2a cells.

Resveratrol induces a downward trend in lactate production by Neuro2a cells

The culture DMEM constituents in the study did not contain lactate (Invitrogen, Catalogue 11960). Lactate accumulated in the media of control cells measured 11.0 ± 0.6 mM and 26.6 ± 1.2 mM after 24 and 48h, respectively. Neuro2a treated with resveratrol showed a slow decrease in lactate release during the first 24 h and a more pronounced decrease in the next 24 h (Table 2 and Table 3). The values of lactate release or glucose production in cells treated with resveratrol were expected to be higher than cell viability (the values of end-point) since the population of cells treated with high concentrations of resveratrol was decreased over time due to the toxicity of the compound. During 48 h treatment, however, the data indicate that resveratrol at the concentrations of 12.5 μ M and 25 μ M caused a reduction in lactate production by the Neuro2a population by around 20% and 29%, respectively whereas the reduction in cell viability was only 3% and 19% (Table 3). Therefore, these results suggest that there was a downward trend in lactate released by cells incubated with resveratrol. One reason could be that resveratrol might improve mitochondrial function; thereby more lactate could enter the next steps (citric acid cycle and oxidative phosphorylation) and produce a boost of ATP. This hypothesis is in agreement with other studies that also reported resveratrol to be able to protect cells from mitochondrial dysfunction and to increase the number of mitochondria [45-47].

Discussion

Energy depletion has been implicated as an early hallmark of AD occurring even prior to clinical symptoms and consistently in association with the progression of the disease in numerous studies [4, 14]. Viewed from this perspective, resveratrol – a natural antioxidant having the capability of stimulating AMPK – was tested for its effects on levels of important nucleotides. Resveratrol showed a strong ability to elevate high energy carrier ATP in the neuronal culture model Neuro2a cell line. This work also provides the new angle of resveratrol in a capability of increasing GTP levels which has not been reported in the literature so far. Although GTP is less abundant than ATP, GTP plays an important role in specific processes including microtubule assembly [48], a defective process found in AD brains [49]. Furthermore, resveratrol reversed a drop in ECP due to nutrition-restricted conditions at the late stages of the experiments, from approximately 60% of untreated cells to higher than 80% even at low concentrations of resveratrol treatment which did not cause significant reduction in cell viability.

At low doses ($< 10\mu\text{M}$), the literature suggests that resveratrol protects cells from mitochondrial dysfunction [1-3], increases the number of mitochondria [4] and induces the disposal of existing dysfunctional mitochondria [5-7]. High doses of resveratrol ($>100\ \mu\text{M}$) induce apoptosis [8], resulting in loss of mitochondrial membrane potential [9, 10].

Furthermore our experiments demonstrated that resveratrol treatment resulted in consistent increases in total adenine nucleotide levels. Since the sum of adenine nucleotides has been found to be markedly reduced in the tissue from AD patients obtained at diagnostic craniotomy compared to control values [15], these findings in our study might provide a promising clue of the effect of resveratrol on the improvement of total adenine nucleotide contents in AD brain *in vivo*.

Energy depletion is likely to play a major role in neurodegenerative disease, including AD and resveratrol may therefore be protective in these instances. However our results suggest that administering the correct dose would be important. Resveratrol has an ability to increase ATP levels at a concentration of 12.5 μM , a concentration which did not affect cell viability. However, currently it would be difficult to achieve precise concentrations of 12.5 μM in human plasma with oral administration. Research on healthy volunteers who administered up to 5 g oral resveratrol/single dose (10 times higher than normal oral dose of resveratrol) indicated that peak plasma levels of resveratrol were only 2.4 μM , occurred 1.5 h post-dose [50]. Therefore, in order to provide concentrations able to increase intracellular ATP levels as such 12.5 μM resveratrol in human plasma, resveratrol given by injection may be required.

Despite resveratrol-induced steady increases in ATP and GTP levels, there is no upward trend found in glucose content consumed by Neuro2a cells and even a substantial reduction in glucose uptake was identified with resveratrol treatment. In combination with the results of lactate production which showed that Neuro2a cells treated with resveratrol had a tendency to decrease lactate release, suggesting the role of resveratrol to restrain aerobic glycolysis – an ineffective way to produce ATP from glucose. The study of Vlassenko et al. [51], conducted on living AD patients and healthy volunteers, indicates that high levels of aerobic glycolysis correlate with the β -amyloid ($\text{A}\beta$) deposition in individuals with AD. Since AD is found to link to aerobic glycolysis *in vivo* [51, 52], compounds able to suppress aerobic glycolysis, such as resveratrol, can be seen as a potential approach for the AD treatment.

In a recent study [53], resveratrol is also demonstrated to reduce both glucose uptake and lactate production, leading to decreased cellular proliferation in various cancer cell lines such as HeLa, HepG2 and MCF-7. Suppression of aerobic glycolysis may account for the

anticancer properties of resveratrol [53] because cancer cell metabolism is characterized by increased glucose uptake and lactate production to provide cancer cells with an advantage to grow even in low-oxygen environments within tumors [54].

If aerobic glycolysis is suppressed, more pyruvate can enter the next metabolic steps where an abundance of ATP can be generated in the mitochondria and no further glucose consumption is required. The powerhouse of energy production, mitochondrial function and number primarily impact on the supply of ATP [55]. Numerous studies in the literature also reported resveratrol protected cells from mitochondrial dysfunction [45-47]. Interestingly, resveratrol was found to increase the number of mitochondria in various cell lines and tissues, such as in Neuro2a cells [28], endothelial cells [56] and muscle [45]. In cultured human coronary arterial endothelial cells, resveratrol up-regulated protein expression of electron transport chain components, enhanced mitochondrial DNA content and mitochondrial mass, activated mitochondrial biogenesis factors (peroxisome proliferator-activated receptor- γ coactivator-1 α PGC-1 α , nuclear respiratory factor-1 NRF-1, mitochondrial transcription factor-A mtTF-A) [56]. Knockdown of NAD⁺-dependent protein deacetylase SIRT1 prevented resveratrol-triggered mitochondrial biogenesis, suggesting that resveratrol elevated mitochondrial content in a SIRT1-dependent manner [56].

In neurons, however, resveratrol stimulated mitochondrial biogenesis in an AMPK-dependent manner through its upstream LKB1 pathway, but independent of SIRT1 [28]. AMPK activation facilitates not only the generation of new mitochondria, but also the destruction of defective mitochondria [57]. Activated AMPK promotes the biogenesis of new mitochondria through effects on PGC-1 α -dependent transcription and simultaneously induces the disposal of existing dysfunctional mitochondria via serine/threonine-protein kinase ULK1-dependent

mitophagy [57-59]; therefore explaining the manipulation of resveratrol in ATP production occurring in the mitochondria. It is well-established that AMPK activation is capable of switching off the ATP consumption and switching on the ATP production in cells. However, in the case of resveratrol, the findings of our experiments also found that it also up-regulates the levels of GTP, apart from ATP.

However, AMPK activation would rather improve energy charge than increase the total nucleotide pool. There are alternative reasons which may account for the increase of the total nucleotide pool caused by resveratrol. Resveratrol has been found to arrest cell growth and cell cycle progression, which are energy-consuming progresses [61, 62]. Resveratrol promotes DNA synthesis inhibition and a strong but reversible S-phase delay by the activation of proteins controlling cell cycle progression cyclin-dependent kinase 2 and checkpoint kinase 2 as well as the inhibition of phosphatidylinositol 3'-kinase/Akt signaling pathway [61]. If the total numbers of cell division are expected to remain constant in a given organism, slowing down of the cell cycle selectively in the S-phase would result in aging-delay of this organism and may explain the effect of resveratrol on lifespan prolongation [61].

In conclusion, a growing body of literature has suggested a possible beneficial role of resveratrol for AD. Experiments on AD transgenic mouse models with oral resveratrol administration showed that A β levels and deposition were reduced in the cortex compared to controls [29]. Resveratrol pronouncedly decreased the amounts of secreted and intracellular A β peptides generated from different cell lines by facilitating intracellular degradation of A β involving the proteasome [63]. In addition, resveratrol can cross through the intact blood-brain barrier to exert its strong antioxidant properties against oxidative stress in AD [64]. Apart from those benefits of resveratrol against amyloid plaques or oxidative stress as

therapeutic targets reported in the literature, this study represents a fruitful new mechanism of resveratrol on improvement of cellular energy levels and supports the potential of low concentrations of resveratrol for the prevention and treatment of AD.

Figure Legends

Fig. 1: Cell viability after resveratrol treatment. Toxicity of resveratrol on cell viability appeared from the concentration of 25 μ M in both 24h and 48h time-points. Values = Mean $\pm$ SEM (3 experiments). Significant differences were assessed by one-way ANOVA with Dunnett's multiple comparison test, * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

Fig. 2: Chromatograms of Neuro2a extracts after treatment with 0 μ M (A), 12.5 μ M (B) and 100 μ M (C) resveratrol at 6 h time-point. The peak areas of each compound were integrated and compared to a standard curve to calculate the individual concentrations of nucleotides.

Fig. 3: Chromatograms of Neuro2a extracts after treatment with 0 μ M (A), 12.5 μ M (B) and 100 μ M (C) resveratrol after 48 h. The peak areas of each compound were integrated and compared to a standard curve to calculate the individual concentrations of nucleotides.

Fig. 4: Intracellular ATP and GTP levels of Neuro2a cells after treatment with resveratrol. ATP (A) and GTP levels (B), normalised to protein contents, were significantly increased in cells treated with resveratrol in all experimental time-points from 1 h to 48 h. Data are the mean $\pm$ SEM of 3 independent experiments in duplicate. Significant differences were assessed by one-way ANOVA with Dunnett's multiple comparison test and shown as * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

Fig. 5: Effect of resveratrol on intracellular ADP (A), AMP (B) and GDP (C) levels of Neuro2a cells. Nucleotide levels were normalised to protein concentration and data are the mean $\pm$ SEM of 3 independent experiments in duplicate. Significant differences were assessed

by one-way ANOVA with Dunnett's multiple comparison test and shown as * $p<0.05$, ** $p<0.01$ and *** $p<0.001$.

Fig. 6: ECP ratios of Neuro2a cells after 1 - 48 h treatment with resveratrol. (A) ECP maintained at the high level over 80% during first 24 h in cells with or without resveratrol addition. (B) At 48 h time-point, ECP fell to under 70% in untreated cells whilst resveratrol suppressed the decrease in ECP and elevated it to over 80%. Values = Mean $\pm$ SEM (3 experiments); * $p<0.05$, ** $p<0.01$ and *** $p<0.001$.

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References

1. Terry, R.D. and P. Davies, *Dementia of the Alzheimer type*. Annual review of neuroscience, 1980. **3**: p. 77-95.
2. Razani, J., et al., *Patterns of deficits in daily functioning and cognitive performance of patients with Alzheimer disease*. Journal of geriatric psychiatry and neurology, 2011. **24**(1): p. 23-32.
3. Mosconi, L., et al., *Early detection of Alzheimer's disease using neuroimaging*. Experimental gerontology, 2007. **42**(1-2): p. 129-38.
4. Haxby, J.V., et al., *Neocortical metabolic abnormalities precede nonmemory cognitive defects in early Alzheimer's-type dementia*. Archives of neurology, 1986. **43**(9): p. 882-5.
5. Sakamoto, S., et al., *Differences in cerebral metabolic impairment between early and late onset types of Alzheimer's disease*. Journal of the neurological sciences, 2002. **200**(1-2): p. 27-32.
6. Salehi, A. and D.F. Swaab, *Diminished neuronal metabolic activity in Alzheimer's disease. Review article*. Journal of neural transmission, 1999. **106**(9-10): p. 955-86.
7. Kapogiannis, D. and M.P. Mattson, *Disrupted energy metabolism and neuronal circuit dysfunction in cognitive impairment and Alzheimer's disease*. Lancet neurology, 2011. **10**(2): p. 187-98.
8. Hanson, R.W., *The role of ATP in metabolism*. Biochemical Education, 1989. **17**(2): p. 86-92.
9. Silver, I.A., J. Deas, and M. Erecinska, *Ion homeostasis in brain cells: differences in intracellular ion responses to energy limitation between cultured neurons and glial cells*. Neuroscience, 1997. **78**(2): p. 589-601.

10. Kann, O. and R. Kovacs, *Mitochondria and neuronal activity*. American journal of physiology. Cell physiology, 2007. **292**(2): p. C641-57.
11. Thomas, R.C., *Electrogenic sodium pump in nerve and muscle cells*. Physiological reviews, 1972. **52**(3): p. 563-94.
12. Du, H., et al., *Early deficits in synaptic mitochondria in an Alzheimer's disease mouse model*. Proceedings of the National Academy of Sciences of the United States of America, 2010. **107**(43): p. 18670-5.
13. Attwell, D. and S.B. Laughlin, *An energy budget for signaling in the grey matter of the brain*. Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism, 2001. **21**(10): p. 1133-45.
14. Hoyer, S., *Oxidative energy metabolism in Alzheimer brain. Studies in early-onset and late-onset cases*. Molecular and chemical neuropathology / sponsored by the International Society for Neurochemistry and the World Federation of Neurology and research groups on neurochemistry and cerebrospinal fluid, 1992. **16**(3): p. 207-24.
15. Sims, N.R., et al., *Metabolic processes in Alzheimer's disease: adenine nucleotide content and production of $^{14}\text{CO}_2$ from $[\text{U-}^{14}\text{C}]$ glucose in vitro in human neocortex*. Journal of neurochemistry, 1983. **41**(5): p. 1329-34.
16. Salminen, A., et al., *AMP-activated protein kinase: a potential player in Alzheimer's disease*. Journal of neurochemistry, 2011. **118**(4): p. 460-74.
17. Fogarty, S. and D.G. Hardie, *Development of protein kinase activators: AMPK as a target in metabolic disorders and cancer*. Biochimica et biophysica acta, 2010. **1804**(3): p. 581-91.

18. Weisova, P., et al., *Role of 5'-adenosine monophosphate-activated protein kinase in cell survival and death responses in neurons*. Antioxidants & redox signaling, 2011. **14**(10): p. 1863-76.
19. Hardie, D.G., *AMP-activated protein kinase: an energy sensor that regulates all aspects of cell function*. Genes & development, 2011. **25**(18): p. 1895-908.
20. Paiva, M.A., et al., *Enhancing AMPK activation during ischemia protects the diabetic heart against reperfusion injury*. American journal of physiology. Heart and circulatory physiology, 2011. **300**(6): p. H2123-34.
21. Hwang, J.T., et al., *Resveratrol protects ROS-induced cell death by activating AMPK in H9c2 cardiac muscle cells*. Genes & nutrition, 2008. **2**(4): p. 323-6.
22. Kubota, N., et al., *Adiponectin stimulates AMP-activated protein kinase in the hypothalamus and increases food intake*. Cell metabolism, 2007. **6**(1): p. 55-68.
23. Minokoshi, Y., et al., *AMP-kinase regulates food intake by responding to hormonal and nutrient signals in the hypothalamus*. Nature, 2004. **428**(6982): p. 569-74.
24. Jager, S., et al., *AMP-activated protein kinase (AMPK) action in skeletal muscle via direct phosphorylation of PGC-1alpha*. Proceedings of the National Academy of Sciences of the United States of America, 2007. **104**(29): p. 12017-22.
25. Sambandam, N. and G.D. Lopaschuk, *AMP-activated protein kinase (AMPK) control of fatty acid and glucose metabolism in the ischemic heart*. Progress in lipid research, 2003. **42**(3): p. 238-56.
26. Viollet, B., et al., *AMP-activated protein kinase in the regulation of hepatic energy metabolism: from physiology to therapeutic perspectives*. Acta physiologica, 2009. **196**(1): p. 81-98.

27. Leonard, S.S., et al., *Resveratrol scavenges reactive oxygen species and effects radical-induced cellular responses*. Biochemical and biophysical research communications, 2003. **309**(4): p. 1017-26.
28. Dasgupta, B. and J. Milbrandt, *Resveratrol stimulates AMP kinase activity in neurons*. Proceedings of the National Academy of Sciences of the United States of America, 2007. **104**(17): p. 7217-22.
29. Vingtdeux, V., et al., *AMP-activated protein kinase signaling activation by resveratrol modulates amyloid-beta peptide metabolism*. The Journal of biological chemistry, 2010. **285**(12): p. 9100-13.
30. Giannattasio, S., et al., *Simultaneous determination of purine nucleotides, their metabolites and beta-nicotinamide adenine dinucleotide in cerebellar granule cells by ion-pair high performance liquid chromatography*. Brain research. Brain research protocols, 2003. **10**(3): p. 168-74.
31. Peterson, J.I. and D.S. Young, *Evaluation of the hexokinase-glucose-6-phosphate dehydrogenase method of determination of glucose in urine*. Analytical biochemistry, 1968. **23**(2): p. 301-16.
32. Rosenberg, J.C. and B.F. Rush, *An enzymatic-spectrophotometric determination of pyruvic and lactic acid in blood. Methodologic aspects*. Clinical chemistry, 1966. **12**(5): p. 299-307.
33. Bradford, M.M., *A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding*. Analytical biochemistry, 1976. **72**: p. 248-54.
34. Atkinson, D.E. and G.M. Walton, *Adenosine triphosphate conservation in metabolic regulation. Rat liver citrate cleavage enzyme*. The Journal of biological chemistry, 1967. **242**(13): p. 3239-41.

35. Sellevold, O.F., P. Jynge, and K. Aarstad, *High performance liquid chromatography: a rapid isocratic method for determination of creatine compounds and adenine nucleotides in myocardial tissue*. Journal of molecular and cellular cardiology, 1986. **18**(5): p. 517-27.
36. Yang, M.S. and R.C. Gupta, *Determination of Energy Charge Potential in the C6 Glioma and the HepG-2 Cell Culture*. Toxicology mechanisms and methods, 2003. **13**(2): p. 97-101.
37. Romano, J.C. and R. Daumas, *Adenosine nucleotide "energy charge" ratios as an ecophysiological index for microplankton communities*. Marine Biology, 1981. **62**(4): p. 281-296.
38. Chih, C.P., P. Lipton, and E.L. Roberts, Jr., *Do active cerebral neurons really use lactate rather than glucose?* Trends in neurosciences, 2001. **24**(10): p. 573-8.
39. Schurr, A., *Lactate: the ultimate cerebral oxidative energy substrate?* Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism, 2006. **26**(1): p. 142-52.
40. Magistretti, P.J. and L. Pellerin, *Cellular mechanisms of brain energy metabolism and their relevance to functional brain imaging*. Philosophical transactions of the Royal Society of London. Series B, Biological sciences, 1999. **354**(1387): p. 1155-63.
41. Warburg, O., *On the origin of cancer cells*. Science, 1956. **123**(3191): p. 309-14.
42. Martin, W. and M. Muller, *The hydrogen hypothesis for the first eukaryote*. Nature, 1998. **392**(6671): p. 37-41.
43. Albers, D.S. and M.F. Beal, *Mitochondrial dysfunction and oxidative stress in aging and neurodegenerative disease*. Journal of neural transmission. Supplementum, 2000. **59**: p. 133-54.

44. Ross, J.M., et al., *High brain lactate is a hallmark of aging and caused by a shift in the lactate dehydrogenase A/B ratio*. Proceedings of the National Academy of Sciences of the United States of America, 2010. **107**(46): p. 20087-92.
45. Lagouge, M., et al., *Resveratrol improves mitochondrial function and protects against metabolic disease by activating SIRT1 and PGC-1alpha*. Cell, 2006. **127**(6): p. 1109-22.
46. Shin, S.M., I.J. Cho, and S.G. Kim, *Resveratrol protects mitochondria against oxidative stress through AMP-activated protein kinase-mediated glycogen synthase kinase-3beta inhibition downstream of poly(ADP-ribose)polymerase-LKB1 pathway*. Molecular pharmacology, 2009. **76**(4): p. 884-95.
47. Ungvari, Z., et al., *Mitochondrial protection by resveratrol*. Exercise and sport sciences reviews, 2011. **39**(3): p. 128-32.
48. Arai, T. and Y. Kaziro, *Role of GTP in the assembly of microtubules*. Journal of biochemistry, 1977. **82**(4): p. 1063-71.
49. Iqbal, K., et al., *Defective brain microtubule assembly in Alzheimer's disease*. Lancet, 1986. **2**(8504): p. 421-6.
50. Boocock, D.J., et al., *Phase I dose escalation pharmacokinetic study in healthy volunteers of resveratrol, a potential cancer chemopreventive agent*. Cancer Epidemiol Biomarkers Prev, 2007. **16**(6): p. 1246-52.
51. Vlassenko, A.G., et al., *Spatial correlation between brain aerobic glycolysis and amyloid-beta (A β) deposition*. Proc Natl Acad Sci U S A, 2010. **107**(41): p. 17763-7.
52. Vlassenko, A.G., T.L. Benzinger, and J.C. Morris, *PET amyloid-beta imaging in preclinical Alzheimer's disease*. Biochim Biophys Acta, 2012. **1822**(3): p. 370-9.

53. Iqbal, M.A. and R.N. Bamezai, *Resveratrol Inhibits Cancer Cell Metabolism by Down Regulating Pyruvate Kinase M2 via Inhibition of Mammalian Target of Rapamycin*. PloS one, 2012. **7**(5): p. e36764.
54. Vander Heiden, M.G., L.C. Cantley, and C.B. Thompson, *Understanding the Warburg effect: the metabolic requirements of cell proliferation*. Science, 2009. **324**(5930): p. 1029-33.
55. Nair, K.S., *Aging muscle*. The American journal of clinical nutrition, 2005. **81**(5): p. 953-63.
56. Csiszar, A., et al., *Resveratrol induces mitochondrial biogenesis in endothelial cells*. American journal of physiology. Heart and circulatory physiology, 2009. **297**(1): p. H13-20.
57. Mihaylova, M.M. and R.J. Shaw, *The AMPK signalling pathway coordinates cell growth, autophagy and metabolism*. Nature cell biology, 2011. **13**(9): p. 1016-23.
58. Egan, D.F., et al., *Phosphorylation of ULK1 (hATG1) by AMP-activated protein kinase connects energy sensing to mitophagy*. Science, 2011. **331**(6016): p. 456-61.
59. Li, L., et al., *Mitochondrial biogenesis and peroxisome proliferator-activated receptor-gamma coactivator-1alpha (PGC-1alpha) deacetylation by physical activity: intact adipocytokine signaling is required*. Diabetes, 2011. **60**(1): p. 157-67.
60. Lambeth, D.O., *What is the function of GTP produced in the Krebs citric acid cycle?* IUBMB life, 2002. **54**(3): p. 143-4.
61. Zhou, R., et al., *Induction of a reversible, non-cytotoxic S-phase delay by resveratrol: implications for a mechanism of lifespan prolongation and cancer protection*. British journal of pharmacology, 2009. **158**(2): p. 462-74.
62. Joe, A.K., et al., *Resveratrol induces growth inhibition, S-phase arrest, apoptosis, and changes in biomarker expression in several human cancer cell lines*. Clinical cancer

- research : an official journal of the American Association for Cancer Research, 2002. **8**(3): p. 893-903.
63. Marambaud, P., H. Zhao, and P. Davies, *Resveratrol promotes clearance of Alzheimer's disease amyloid-beta peptides*. The Journal of biological chemistry, 2005. **280**(45): p. 37377-82.
64. Darvesh, A.S., et al., *Oxidative stress and Alzheimer's disease: dietary polyphenols as potential therapeutic agents*. Expert review of neurotherapeutics, 2010. **10**(5): p. 729-45.