Hypochlorite-induced structural modifications enhance the chaperone activity of human α2-macroglobulin

Amy Wyatt  
*University of Wollongong*, awyatt@uow.edu.au

Janet Kumita  
*University of Cambridge*

Richard W. Mifsud  
*University of Cambridge*

Cherrie A. Gooden  
*University of Wollongong*, cgooden@uow.edu.au

Mark Wilson  
*University of Wollongong*, mrw@uow.edu.au

*See next page for additional authors*

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Abstract
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but the direct activation of mammalian chaperones by hypochlorite has not, to our knowledge, been 
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Moreover, dimeric α2M is generated in whole-blood plasma in the presence of physiologically relevant 
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stable soluble complexes with misfolded client proteins, including heat-denatured enzymes, oxidized 
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hypochlorite-modified α2M delivers its misfolded cargo to lipoprotein receptors on macrophages and 
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Keywords
Molecular chaperone, inflammation, protein folding, clearance, CMMB

Disciplines
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Authors
Amy Wyatt, Janet Kumita, Richard W. Mifsud, Cherrie A. Gooden, Mark Wilson, and Christopher M. Dobson

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Hypochlorite-induced structural modifications enhance the chaperone activity of human α₂-macroglobulin

Amy R. Wyatt⁴ᵇ, Janet R. Kumita⁴, Richard W. Mifsud⁴, Cherrie A. Gooden⁴, Mark R. Wilson⁴ᵇ,¹, and Christopher M. Dobson⁴ᵃ,¹

¹Department of Chemistry, University of Cambridge, Cambridge CB2 1EW, United Kingdom; and ²Illawarra Health and Medical Research Institute and School of Biological Sciences, University of Wollongong, Wollongong, NSW 2522, Australia

¹To whom correspondence may be addressed. E-mail: mrw@uow.edu.au or cmd44@cam.ac.uk.

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Significance

Hypochlorite, an oxidant generated in vivo by the innate immune system, kills invading pathogens largely by inducing the misfolding of microbial proteins. Concomitantly, the nonspecific activity of hypochlorite also damages host proteins, and the accumulation of damaged (misfolded) proteins is implicated in the pathology of a variety of debilitating human disorders (e.g., Alzheimer’s disease, atherosclerosis, and arthritis). In the present study, we show that the chaperone activity of human α₂-macroglobulin, a highly abundant secreted protein, is dramatically increased by hypochlorite-induced structural modifications. The data support the conclusion that α₂-macroglobulin is a unique component of the innate immune system that is posttranslationally regulated by hypochlorite to facilitate the clearance of potentially pathogenic misfolded proteins.


The authors declare no conflict of interest.

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Abstract

Hypochlorite, an oxidant generated in vivo by the innate immune system, kills invading pathogens largely by inducing the misfolding of microbial proteins. Concomitantly, the nonspecific activity of hypochlorite also damages host proteins, and the accumulation of damaged (misfolded) proteins is implicated in the pathology of a variety of debilitating human disorders (e.g., Alzheimer’s disease, atherosclerosis, and arthritis). It is well-known that cells respond to oxidative stress by up-regulating proteostasis machinery, but the direct activation of mammalian chaperones by hypochlorite has not, to our knowledge, been previously reported. In this study, we show that hypochlorite-induced modifications of human α₂-macroglobulin (α₂M) markedly increase its chaperone activity by generating species, particularly dimers formed by dissociation of the native tetramer, which have enhanced surface hydrophobicity. Moreover, dimeric α₂M is generated in whole-blood plasma in the presence of physiologically relevant amounts of hypochlorite. The chaperone activity of hypochlorite-modified α₂M involves the formation of stable soluble complexes with misfolded client proteins, including heat-denatured enzymes, oxidized fibrinogen, oxidized LDL, and native or oxidized amyloid β-peptide (Aβ₄₂). Here, we show that hypochlorite-modified α₂M delivers its misfolded cargo to lipoprotein receptors on macrophages and reduces Aβ₄₂ neurotoxicity. Our results support the conclusion that α₂M is a specialized chaperone that prevents the extracellular accumulation of misfolded and potentially pathogenic proteins, particularly during innate immune system activity.

Keywords: molecular chaperone | inflammation | protein folding | clearance

Introduction

Hypochlorite, a potent oxidant produced by immune cells through the myeloperoxidase-H₂O₂-chloride system, kills invading microbes predominately by inducing the misfolding and aggregation of their proteins (1). The effects of hypochlorite, however, are nonspecific; therefore, when generated in vivo, the host organism suffers collateral damage...
α2-Macroglobulin (α2M) is a highly abundant secreted protein that is best known for its ability to trap proteases (9); α2M can, however, interact with a broad range of molecules, and consequently, many other biological roles have been suggested, including targeting cytokines for clearance, sequestration of zinc, and opsonization of bacteria (reviewed in ref. 10). Furthermore, α2M has been identified as one of a small number of abundant extracellular chaperones (11). Although our understanding of extracellular proteostasis has increased in recent years, it is still limited compared with our extensive knowledge of the mechanisms comprising intracellular proteostasis (12). Several in vitro studies have shown that α2M can inhibit both fibril formation by amyloidogenic proteins and peptides (13–16) and stress-induced amorphous aggregation of other systems (17). In addition, recent studies have shown that α2M can protect neurons from toxic oligomers of the protein HypF-N and the Alzheimer’s disease-relevant amyloid β-peptide (Aβ1–42) by preventing them from binding to the cell surface (18, 19). This action is implicated in the ability of α2M to inhibit inflammatory responses of glia induced by Aβ1–42 in vitro and in vivo (19). α2M is also found colocalized with misfolded protein deposits in Alzheimer’s disease (20), the spongiform encephalopathies (21), atherosclerosis (22), and arthritis (23), reflecting the broad importance of its biological role as an extracellular chaperone.

α2M is composed of four identical subunits that are paired by disulfide bonds to form covalently linked dimers; these dimers then noncovalently associate to complete the 720 kDa tetramer (24). It has been shown previously that α2M is highly sensitive to the effects of hypochlorite, which induces dissociation of native α2M tetramers into stable dimers that are no longer able to trap proteases (25–27). In contrast, exposure of α2M to hypochlorite enhances its binding to some cytokines and growth factors and reveals on α2M the cryptic binding site for the endocytic low-density lipoprotein receptor-related protein (LRP) (28, 29). Thus, the results of previous studies suggest that modification by hypochlorite could serve as a switch to control the functions of α2M during innate immune system activity. Given that hypochlorite is a potent inducer of protein misfolding and aggregation, in the current study, we have investigated the effect of hypochlorite on the chaperone activity of α2M using a range of model and disease-relevant client proteins.

Results
Hypochlorite-Induced Modification of α2M Increases Its Surface Hydrophobicity. Incubation of purified human α2M with sodium hypochlorite (NaOCl) results in a dose-dependent dissociation of the native tetramer into dimers (Fig. 1A). After treatment with NaOCl, the remaining α2M tetramer species migrate similarly to the native protein and are not converted to the more compact “fast” conformation (which results from nucleophilic attack of the α2M thioester bond) (30). The α2M dimers liberated by NaOCl treatment are stable, they do not readily form tetramers or any other higher molecular mass species when stored for several months at 4 °C, and they can be purified by size exclusion chromatography (SEC).

By Western blot analysis, α2M is predominantly detected in its native tetrameric form in human blood plasma (Fig. 1B). As observed for experiments using purified α2M (Fig. 1A), dimeric α2M is liberated after incubating the plasma with NaOCl in a dose-dependent manner (Fig. 1B). Notably, dimeric α2M can be detected in blood plasma in the presence of micromolar to millimolar concentrations of NaOCl, which are predicted to be physiologically relevant based on in vitro and ex vivo studies (2, 31–34). Diluting blood plasma with PBS (1:4 or 1:100) to give a final protein concentration similar to that of interstitial fluid or cerebral spinal fluid, respectively, results in greater amounts of the α2M dimers being liberated by the same concentration of NaOCl (Fig. 1C). This effect is a consequence of increasing the ratio of NaOCl to its protein targets.

CD spectroscopy shows that the secondary structure of purified α2M remains relatively unchanged after incubation with 12.5 μM NaOCl (Fig. 1D), which dissociates ∼25% of the α2M into dimers (Fig. 1A). This result suggests that extensive unfolding is not required to induce dissociation of the tetramer; however, at higher concentrations of NaOCl, the dissociation of α2M into dimers is accompanied by a significant loss of secondary structure. 8-Anilino-1-naphthalenesulfonic acid (ANS) fluorescence measurements indicate that there is a dose-dependent increase in the surface hydrophobicity of α2M after treatment with concentrations of NaOCl up to 50 μM but that there is no additional significant increase after treatment with 75 μM NaOCl (Fig. 1E). Given that ∼90% of α2M is converted to dimers after incubation with 50 μM NaOCl (Fig. 1A), the results suggest that the physical dissociation of α2M tetramers to dimers is the major structural
change responsible for enhanced ANS binding. Hereafter, the term oxidized is used to describe α2M that has been treated with NaOCl. When ANS binding to isolated oxidized α2M dimer or tetramer (purified by SEC) is measured, both species have increased surface hydrophobicity compared with the native tetramer (Fig. 1F); however, the oxidized α2M dimer has around fourfold greater surface hydrophobicity than the oxidized α2M tetramer. These findings are consistent with previous reports that the protein surface juxtaposed between noncovalently associated α2M subunits contains hydrophobic regions (35, 36). For the oxidized α2M tetramer, it is unclear whether the enhanced surface hydrophobicity is a result of the partial opening of the dimer interface or other structural changes.

Hypochlorite-Induced Structural Modifications Enhance the Chaperone Activity of α2M. It is likely that, under conditions where α2M is modified by hypochlorite in vivo, oxidative stress will be the major force driving the aggregation of misfolded client proteins. However, to examine in isolation the effects of oxidation of α2M on its chaperone activity and avoid potential complications introduced by the antioxidant effects of the high methionine content of α2M (37), we initially tested the ability of oxidized α2M (i.e., α2M pretreated with NaOCl) to inhibit heat-induced protein aggregation. Creatine phosphokinase (CPK) and citrate synthase (CS) are experimentally convenient model proteins that misfold and aggregate in response to relatively mild heat stress (43 °C). Although native α2M inhibits the heat-induced aggregation of CPK and CS at 43 °C, this process is relatively inefficient and typically requires a near equimolar ratio of the chaperone to the client to have an appreciable effect (Fig. 2 A and B). After exposure to NaOCl (and removal of any unreacted oxidant), oxidized α2M much more potently inhibits the heat-induced aggregation of CPK and CS (Fig. 2 C and D, respectively). This effect is dependent on the concentration of NaOCl used to treat α2M and reaches a maximum when most of the α2M is in the form of dimers. Even at the lowest concentration of NaOCl tested (12.5 μM), when used at molar ratios of α2M:CPK and α2M:CS of ~1:9 and 1:5, respectively, oxidized α2M reduces the final level of client protein turbidity by about one-third compared with little effect from equivalent ratios of native α2M. After incubation of native α2M with 15 μM NaOCl, α2M tetramers and dimers were purified by SEC, and their relative chaperone activities were compared in similar protein aggregation assays. The comparisons indicate that all oxidized α2M species have enhanced chaperone activity compared with that of native α2M; however, the dimer liberated after oxidation is a more potent chaperone than the oxidized tetramer (Fig. 2 E and F). Taken together with the results of ANS binding assays (Fig. 1 E and F), these data indicate that the level of chaperone activity correlates with the level of surface-exposed hydrophobicity on α2M species (i.e., native tetramer < oxidized tetramer < oxidized dimer).

Although nonphysiological elevated temperatures (≥60 °C) induce the dissociation of α2M into dimers, prolonged incubation at 43 °C does not have this effect, regardless of whether or not α2M has been pretreated with NaOCl (Fig. S1A). Moreover, solutions of native or oxidized α2M alone do not increase in turbidity when heated at 43 °C (Fig. S1B). When the control protein bovine serum albumin (BSA) is coincubated with CPK or CS (instead of α2M), there is little effect on their heat-induced aggregation, regardless of whether or not BSA is pretreated with NaOCl (Fig. S1 C and D). To determine whether or not oxidation is the only treatment that can increase the chaperone activity of α2M, we used sodium thiocyanate (an amine-modifying reagent previously shown to induce dissociation of α2M into dimers) (35) to generate α2M dimers and then, show that they have significantly increased chaperone activity relative to native α2M (Fig. S2). This result confirms that the dissociation of α2M into dimers and the associated increase in chaperone activity are not exclusively dependent on oxidation but can be accomplished by other types of chemical modification.

α2M Forms Complexes with Fibrinogen and LDL Particles in the Presence of Hypochlorite. Fibrinogen (FGN) and apolipoprotein B-100 (the major protein component of LDL particles) are two examples of extracellular proteins that are implicated in disease when oxidized in vivo (4, 6, 38). As has been shown for many other proteins, incubation of FGN or LDL with hypochlorite induces their self-aggregation into high-molecular mass complexes (1, 39, 40). In contrast, α2M is able to withstand high concentrations of hypochlorite without self-aggregating (Fig. S3). Using a biotin-streptavidin pull-down assay, under native conditions, no detectable α2M was found to coprecipitate with biotinylated LDL (bLDL), and only a small amount of α2M coprecipitates with biotinylated FGN (bFGN); however, coincubation of the proteins in the presence of hypochlorite clearly enhances the amount of α2M that coprecipitates with bLDL and bFGN (Fig. 3A). Regardless of its oxidation state, there is negligible nonspecific binding of α2M to the streptavidin-coated beads, confirming that coprecipitation of α2M only results when it binds to bFGN or bLDL. In separate experiments, we have confirmed that the noncovalent binding of α2M to native bFGN is not significantly enhanced by preoxidation of α2M with hypochlorite (Fig. S4). Although it is conceivable that, in the presence of NaOCl, α2M may become covalently linked to bFGN or bLDL, this possibility is excluded by the observation that, after heating and reduction, the bound α2M migrates at positions corresponding to its dimeric form (360 kDa; which is the result of oxidative intersubunit cross-linking) (27) or its monomeric form (180 kDa) or as heat-liberated fragments (41), and other species are not detected. Furthermore, relative
to native α2M, preoxidized α2M preferentially associates with preoxidized bFGN and preoxidized bLDL, indicating that the oxidized proteins bind through noncovalent interactions (Fig. 3B).

**Hypochlorite-Induced Structural Modifications Enhance the Ability of α2M to Inhibit AB1–42 Aggregation.** Native α2M has previously been shown to inhibit the fibrillar aggregation of amyloidogenic proteins and peptides in vitro (14). To determine if modification by hypochlorite influences the ability of α2M to inhibit this type of process, we examined the effects of native and oxidized α2M on the aggregation of the Alzheimer’s disease-relevant AB1–42 peptide. A thioflavin-T (Th-T) assay was used to monitor the kinetics of fibril formation as described previously (14). Under the conditions used here, when present at a molar ratio of α2M:AB1–42 of 1:25, native α2M extends the lag phase of AB1–42 aggregation from 50 to 80 min (Fig. 4A). An equivalent quantity of oxidized α2M (i.e., α2M pretreated with 20 μM NaOCl) extends it to 100 min, and the lag phase is further extended to 150 min when α2M is more highly oxidized (i.e., pretreated with 50 μM NaOCl). The reduction in the intensity of the Th-T signal is concurrent with a decrease in fibrillar species as detected by transmission electron microscopy (TEM) (Fig. 4B).

Native α2M does not bind detectably to monomeric AB1–42 (42) but inhibits AB1–42 aggregation by binding to soluble oligomers formed early along the aggregation pathway (14). In biotin-streptavidin pull-down assays, α2M binds to biotinylated AB1–42 (bAB1–42) when the former has previously been oxidized (Fig. 5A). Interestingly, this interaction is markedly increased when bAB1–42 is also pretreated with NaOCl, and indeed, native α2M was detected coprecipitating with NaOCl-treated bAB1–42 (i.e., oxidized bAB1–42) but not native bAB1–42 (Fig. 5A). Thus, hypochlorite-induced modification of either α2M or bAB1–42 seems to influence the subsequent binding interactions. When oxidized α2M is incubated with AB1–42 under nonoxidizing conditions and then subjected to native gel electrophoresis, AB1–42 is detected by Western blotting as migrating to positions corresponding to those of oxidized α2M tetramers and dimers (Fig. 5B). Consistent with these results from biotin-streptavidin pull-down assays, the binding of AB1–42 to α2M is greatest when both AB1–42 and α2M are oxidized.

Analysis by Western blotting after separation by denaturing gel electrophoresis shows that AB1–42 forms soluble, SDS-resistant oligomers when treated with NaOCl in the absence of α2M (Fig. 5C). Given that native α2M has been shown to bind preferentially to aggregated AB1–42 (14), the presence of these oligomeric species is a likely explanation for the enhanced binding of oxidized AB1–42 to α2M. Taken together, our results support the conclusion that hypochlorite modification of α2M increases its ability to bind to native and aggregated forms of AB1–42 and in this way, increases its ability to inhibit AB1–42 aggregation. Similar experiments that show the preferential formation of complexes between oxidized α2M and heat-denatured biotinylated CPK are given in Fig. 55.

**Formation of Complexes Between AB1–42 and Oxidized α2M Increases the Binding of AB1–42 to Macrophage Lipoprotein Receptors and Reduces AB1–42 Neurotoxicity.** Because the hypochlorite-induced structural modifications of α2M can expose the LRP binding site (28) and also influence the binding of α2M to LRP (Fig. 5A and B), we examined the effect of preincubating bAB1–42 with native or oxidized α2M on the subsequent binding of bAB1–42 to RAW 264.7 macrophages. Given that there is a dose-dependent effect of hypochlorite on the binding of oxidized α2M to LRP (28), we established that incubation of α2M with 25 μM NaOCl produces the greatest binding to lipoprotein receptors and used this treatment to prepare oxidized α2M for our in vitro assays (Fig. 56). Incubation of RAW 264.7 cells with bAB1–42 alone results in measurable binding of the peptide to the cell surface (Fig. 6A). Preincubation of the cells with receptor-associated protein (RAP), a pan-specific ligand of lipoprotein receptors, including LRP, inhibits about 30% of the total cell surface binding of bAB1–42, implying that this fraction of the binding is lipoprotein receptor-dependent (LR-D). This finding is consistent with previous reports showing that AB1–42 binds directly to LRP in vitro (43); however, the result also suggests that the binding of bAB1–42 alone to the RAW 264.7 cells occurs predominately through lipoprotein receptor-independent (LR-I) mechanisms (i.e., through other receptors or directly to the cell membrane).

Preincubation of bAB1–42 with native α2M significantly reduces the subsequent binding of bAB1–42 to RAW 264.7 cells (Fig. 6A). This effect is likely to be because of native α2M sequestering bAB1–42 in complexes rather than competing for the same cell surface binding site(s), because preincubation of RAW 264.7 cells with native α2M does not affect the subsequent cell surface binding of bAB1–42 alone (Fig. 6B). These results are consistent with the results of a previous study showing that coincubation of preformed AB1–42 oligomers with native α2M inhibits the subsequent binding of the oligomers to cells (19). Because the binding of native α2M to oxidized, oligmeric bAB1–42 is substantially greater than to monomeric bAB1–42 (Fig. 5A), the ability of native α2M to inhibit bAB1–42 cell surface binding suggests that oligmeric bAB1–42 species may be present and that they bind preferentially to the cell surface. Examination of the composition of untreated bAB1–42 peptide samples confirms that a small population of oligomers is present at the start of the assay (Fig. 57). These oligomers and possibly others formed during the course of the experiment may be responsible for the fraction of bAB1–42 cell surface binding that
is inhibited by preincubation of the peptide with native α2M (around 75% of the total binding) (Fig. 5A). Analysis of the cell surface binding of bAβ1–42 after pretreatment of the cells with RAP indicates that coinoculation of native α2M and bAβ1–42 inhibits the subsequent binding of bAβ1–42 to RAW 264.7 cells through LR-I mechanisms but not the smaller fraction of binding mediated by LR-D mechanisms (Fig. 5A).

When bAβ1–42 is incubated with α2M that has been preoxidized with 25 μM NaOCl (conditions that enhance the binding of α2M to lipoprotein receptors) (28), the total binding of bAβ1–42 is not significantly different from that of bAβ1–42 alone (Fig. 6A). However, preincubation of bAβ1–42 with oxidized α2M increases the LR-D binding of bAβ1–42 to RAW 264.7 cells from about 30% to 80% of the total and significantly decreases its LR-I binding. As for native α2M, preincubation of the cells with oxidized α2M does not affect the subsequent binding of bAβ1–42 alone (Fig. 6B). Thus, collectively, the data suggest that the enhanced LR-D binding of bAβ1–42 results from the formation of soluble oxidized α2M–bAβ1–42 complexes, which bind to RAW 264.7 cells through LR-D but not LR-I mechanisms.

Having observed that oxidized bAβ1–42 (enriched in SDS resistant oligomers) (Fig. 5C) binds more strongly to native and oxidized α2M than untreated bAβ1–42 (Fig. 5 A and B), we repeated the cell binding experiments using oxidized bAβ1–42. Consistent with the idea that oligomeric bAβ1–42 binds readily to the surface of RAW 264.7 cells, treatment of bAβ1–42 with hypochlorite markedly increases its binding to RAW 264.7 cells (Fig. 6C), and only about 20% of the oxidized bAβ1–42 cell surface binding is attributable to LR-D mechanisms (Fig. 6D). Similar to the results for native bAβ1–42, preincubating oxidized bAβ1–42 with native α2M inhibits the subsequent binding of the peptide to RAW 264.7 cells through LR-I but not LR-D mechanisms. Compared with the effects of native α2M, when oxidized bAβ1–42 is preincubated with oxidized α2M, LR-I binding of the peptide is more potently inhibited, and its LR-D binding is coincidently increased (Fig. 6D). In additional experiments, we show that the binding to lipoprotein receptors of a client protein that forms amorphous aggregates is also significantly enhanced after forming soluble complexes with oxidized α2M (Fig. S5).

It has previously been shown that native α2M protects neurons from Aβ1–42 toxicity in vitro and in vivo (19). We find that, when tested at a molar ratio of α2M:β1–42 of 1:150, oxidized α2M, but not native α2M, significantly protects SH-SYSY neuroblastoma cells from Aβ1–42 toxicity (Fig. 6E). When present at higher concentrations, both native α2M and oxidized α2M are able to provide significant protection in these assays (Fig. S8). It is important to note that our oxidized α2M preparation (generated by treatment with 25 μM NaOCl) contains a heterogeneous mixture of species, including ~25% α2M dimers and 75% α2M tetramers (Fig. 1A). As such, it is likely that only a small fraction of the total oxidized α2M is contributing to the enhanced neuroprotective effect that is measured in these assays.

Discussion

It is well-established that molecular chaperones are vitally important for targeting misfolded proteins for repair or degradation within cells (12), and it is becoming increasingly apparent that extracellular chaperones, including α2M, also participate in essential proteostasis systems that function outside of cells (11). It has been shown that hypochlorite generated by stimulated neutrophils induces dissociation of α2M in vitro (26), and increased oxidation of α2M has been detected in rheumatoid arthritis and Alzheimer’s disease (27, 44), two protein misfolding conditions in which myeloperoxidase activity is known to be elevated (5, 45). Furthermore, our experiments in whole-blood plasma confirm that, in complex biological fluids, α2M is readily induced to dissociate into dimers by low, physiologically relevant hypochlorite concentrations (Fig. 1B). Taken together, the results of these studies strongly support the conclusion that hypochlorite-modified α2M, analogous to that used in this study, is generated in vivo. Here, we show that the chaperone activity of α2M is markedly enhanced by hypochlorite-induced structural modification, a process that, therefore, represents a rapid and direct mechanism of increasing extracellular chaperone activity during inflammation, a state that promotes protein misfolding. Given the large number of diseases in which extracellular protein misfolding and inflammatory pathology coexist (46), the significance of the findings in this report may be far-reaching. To our knowledge, no comparable mechanism for mammalian chaperone activation has previously been described.

Significance of Hypochlorite-Activated Chaperones. It is extremely difficult to measure directly the concentrations of hypochlorite generated by the myeloperoxidase-H2O2-chloride system in vivo; however, considering that 106 stimulated neutrophils can produce around 17 nmol hypochlorite in vitro (47) and at sites of inflammation, neutrophil numbers may exceed 5 × 104 cells/mm3 (48), it is feasible that physiological hypochlorite concentrations reach high-micromolar or low-millimolar levels in inflamed tissues (2, 31). Consistent with the idea that a large amount of hypochlorite may be generated in vivo during inflammation, extracellular myeloperoxidase is also estimated to reach millimolar concentrations (33), and using computational modeling, it is predicted that proteins from atherosclerotic plaques are exposed to a 20-fold molar
excess of hypochlorite over their lifetime (32). In addition, it is very clear that proteins are modified by hypochlorite in vivo and that the extent of these modifications is increased in many disease states, including atherosclerosis (40), Alzheimer’s disease (5), kidney disease (49), rheumatoid arthritis (45), osteoarthritis (50), and chronic lung disease (51).

Although protein oxidation may be a consequence of disease, there is evidence to suggest that oxidized proteins may play a causative role in disease progression. The most extensively studied examples include oxidized LDL and oxidized FGN, both of which have many proatherogenic properties in vitro (reviewed in refs. 52 and 53). In this study, we observed that modification of Aβ1–42 by hypochlorite results in the formation of SDS-resistant oligomers that show enhanced noncovalent binding to α2M and also, to the surface of macrophage cells. A great deal of attention has been given to the role of peroxide-mediated and metal-catalyzed oxidative stress in Alzheimer’s disease (54), but far less is known about the possible role of hypochlorite. Although detailed characterization of the effect of hypochlorite on Aβ1–42 is outside the scope of the current study, the high expression of myeloperoxidase in the brains of sufferers of Alzheimer’s disease and the localization of this enzyme in amyloid plaques suggest that hypochlorite oxidation of Aβ1–42 is likely to occur in vivo (5).

In an attempt to evade the innate immune system, bacteria have evolved a highly specialized holdase chaperone, Hsp33, that is rapidly activated on reaction with hypochlorite (1). Intuitively, a corresponding mammalian system would be extremely beneficial to protect host organisms from accumulating damaged proteins during innate immune responses. Therefore, the central observation of this study, that exposure to hypochlorite triggers dissociation of native α2M tetramers into dimers with strongly enhanced chaperone activity, is likely to be of considerable physiological importance. Although several chaperones have been shown to maintain their activity under conditions of oxidative stress (17, 55, 56), there are currently only a small number of reports describing increased mammalian chaperone activity as a result of posttranslational oxidative modification (57–59), and hypochlorite-mediated activation of mammalian chaperones has not, to our knowledge, been previously reported. This observation is significant, because hypochlorite is a much more potent inducer of protein misfolding and aggregation than other physiologically relevant oxidants, such as hydrogen peroxide, a property attributed to disparity between different oxidants in the rates of their reactions with amino acid side chains (1).

Possible Mechanism of Increased Chaperone Activity. Although the chaperone activities of both Hsp33 and α2M are regulated by hypochlorite, they are achieved through very different mechanisms. The intracellular environment where Hsp33 resides is normally reducing, which means that hypochlorite-induced changes are reversed after oxidative stress has subsided (1). In contrast, secreted proteins, such as α2M, exist in an environment that is constantly oxidizing (60); therefore, α2M must respond to increased oxidative stress rather than to a transient departure from a reduced state. Hypochlorite-induced dissociation of α2M is largely caused by methionine oxidation (25). Methionine sulfoxide reductases can reverse the oxidation of methionine residues in vivo; in humans, however, methionine sulfoxide reductases are localized within intracellular compartments and do not seem to be actively secreted (61, 62). Additionally, hypochlorite-induced activation of α2M chaperone activity is accompanied by covalent modifications in the form of dityrosine cross-linking (Fig. S9). Taken together, the available data support the conclusion that hypochlorite activation of α2M chaperone activity is irreversible, and as such, the fates of hypochlorite-modified α2M and its misfolded protein cargo are likely to be LRP-facilitated endocytosis and lysosomal degradation. The latter is analogous to the fate of the fast form of α2M, which also binds to LRP (30).

Each α2M tetramer contains 100 methionine residues, a feature underlying its sensitivity to hypochlorite-induced modification. Thus, even in complex biological solutions (e.g., blood plasma that contains an excess of potential protein targets), α2M dimers are generated in the presence of micromolar concentrations of hypochlorite (Fig. 1 B and C). It is important to note that the single addition of hypochlorite to blood plasma, as performed in this study, is likely to result in its rapid consumption by oxidation reactions; therefore, the stress imposed may be considerably less than that observed at sites of chronic inflammation when the production of hypochlorite is persistent. It has previously been reported that oxidation of 14 methionine residues and a single tryptophan residue results in the dissociation of tetrameric α2M into dimers (25). The exact positions of these residues have yet to be identified; however, we predict the central part of each α2M subunit containing the bait region (i.e., the target for proteolytic cleavage located between amino acid residues 666 and 706 in human α2M) to be important given that point mutations within the bait region have been shown to disrupt the noncovalent binding of α2M dimers (63). The bait region of human α2M is rich in methionine residues (i.e., M666, M673, M688, M697, and M713 located on each human α2M monomer; the latter is located just beyond the bait region), and in the intact tetramer, these methionine residues are closely aligned at the interface between the noncovalently associated α2M dimers (64).
It is thermodynamically favorable for misfolded proteins to aggregate when normally buried hydrophobic regions become exposed to the surrounding aqueous environment (46). Dissociation of tetrameric α2M into dimers results in markedly enhanced exposure of hydrophobicity at the surface of the molecule; however, oxidized α2M does not readily self-aggregate. The precise reasons why oxidized α2M does not self-aggregate are unknown, although one possibility is that the newly exposed hydrophobic regions on oxidized α2Mdimers are maintained in a pocket that was formerly one-half of the central cavity of tetrameric α2M (64). In any case, the results from this study support the idea that human α2M is induced to adopt a dimeric form in the presence of physiologically relevant hypochlorite concentrations, and that this mechanism is specifically designed to enhance its chaperone activity and ability to bind to LRP rather than the latter effects being a random consequence of oxidation.

After exposure to hypochlorite the α2M tetramer, and to a significantly greater extent the α2M dimer, show increased exposed hydrophobicity and chaperone activity (Figs. 1F and 2E and F). Similarly, hypochlorite-modified α2M tetramers and dimers both show increased binding to IL-2 and IL-6 (29), and treatment of α2M with detergent produces tetramers and dimers that bind more strongly to the amyloidogenic protein β2-microglobulin than does native α2M (16). Partial opening of the dimer interface to expose the relevant binding site(s) on tetrameric α2M or the formation of α2M tetramers from modified, dissociated dimers may explain our results and the results of the aforementioned studies, although it is also possible that other structural changes are responsible. We have further shown that α2M dimers generated by treatment with NaCSN also have enhanced chaperone activity (Fig. S2), indicating that physical dissociation from a tetramer to a dimer is sufficient to increase chaperone activity, independent of any requirement for oxidation. Notably, it has been shown that α2M dimers generated by treatment with NaCSN also bind to LRP (35). Therefore, if the dissociation of native α2M into dimers is sufficient to enhance its chaperone activity, its binding to cytokines, and its clearance through LRP, it may be possible to exploit this effect in the development of novel therapeutics.

**Role of α2M in Inflammation**. Inflammation is a state in which many stresses capable of inducing protein misfolding and aggregation are elevated; therefore, it is likely that proteostasis mechanisms will be enhanced during inflammatory events. Moreover, it has been shown that many acute-phase proteins are susceptible to stress-induced misfolding and major endogenous clients for holdase chaperones in human blood plasma (65–67). To survive periods of increased physiological stress, cells use several different strategies to prevent the accumulation of misfolded proteins (68–71). In this study, we show that hypochlorite-induced structural modifications enhance the ability of α2M to inhibit the amorphous aggregation of globular proteins and the fibrillar aggregation of Aβ1–42. After their binding to oxidized α2M, misfolded client proteins are directed to macrophage lipoprotein receptors, which are a potential route for their clearance and degradation. It has been suggested that hydrophobicity is universally recognized as a damage-associated pattern by the innate immune system (72), and consistent with this idea, many studies have shown that misfolded proteins/peptides, whether oxidized or induced to aggregate by other means, have the ability to stimulate innate immune cells and induce proinflammatory responses (52, 53, 73–80). Therefore, the enhanced chaperone activity of oxidized α2M may play an important role in preventing protein misfolding and inflammation from developing into an unregulated and self-perpetuating pathologic cycle. Taken together with the finding that hypochlorite modification of α2M increases its binding to proinflammatory factors (TNF-α, IL-2, IL-6, and basic fibroblast growth factor) and decreases its binding to antiinflammatory factors (TGF-β1 and TGF-β2) (29), the available data support the idea that α2M is a specialized component of the innate immune system that is posttranslationally regulated by hypochlorite production to minimize self-damage associated with acute or chronic inflammation.

**Experimental Procedures**

**Purification and Oxidation of α2M**. Blood plasma used in this study was donated by healthy consenting volunteers under the approval of the University of Wollongong and Illawarra Shoalhaven Local Health District Health Medical Human Ethics Committee (HEO2/080). α2M was purified from human plasma as previously described (13). Oxidized α2M was prepared by adding various amounts of NaOCl to α2M [690 nM in phosphate buffered saline (pH 7.4); PBS; Thermo Scientific] and incubating overnight at room temperature (RT) unless otherwise specified. Using this method, significant quantities of α2M dimers are generated within minutes (Fig. S10). Nevertheless, we used a prolonged incubation period to ensure that (i) the reaction had reached completion and (ii) we could very reproducibly produce hypochlorite-modified α2M preparations containing equivalent amounts of dimers and tetramers. α2M preparations were subsequently dialyzed against PBS or desalted using Zeba Spin columns (Thermo Scientific). For simplicity, the molar concentrations of α2M reported here are calculated based on the intact 720 kDa tetramer, regardless of its dissociation into dimers. The concentrations of hypochlorite in the stock solutions were determined by absorbance spectroscopy (81). In the figures, the standard
nomenclature for hypochlorite-modified α₂M is ox followed by the micromolar concentration of NaOCl used to treat the protein (e.g., ox25 denotes α₂M incubated with 25 μM NaOCl).

**Electrophoresis.** Proteins were subjected to native gel electrophoresis using NuPAGE Novex 3–8% Tris-acetate gels and Novex Tris-glycine native buffer (Life Technologies). Denaturing gel electrophoresis was performed using NuPAGE Novex 4–12% gels and NuPAGE Mes SDS running buffer. Where specified, NuPAGE sample-reducing agent was used to reduce protein samples. CD Spectroscopy. CD spectra were obtained using a Jasco J-810 spectropolarimeter at 25 °C using a 1-mm path-length cuvette. For CD spectroscopy, α₂M (190 nM) was prepared in 10 mM sodium phosphate buffer (pH 7.4).

**ANS Binding Assay.** Native or oxidized α₂M (200 nM) were incubated with ANS (360 μM) in PBS (5 min at RT) before monitoring fluorescence emission at 475 nm (Aex = 350 nm) with slit widths set at 5 nm on a Cary Eclipse spectrofluorimeter (Agilent Ltd.).

**Precipitation Assays.** CPK (Sigma-Aldrich) was incubated in the presence or absence of native or oxidized α₂M (prepared as described above) in PBS and heated at 43 °C in a FLUOstar OPTIMA platereader (BMG Labtech Ltd.) while the absorbance at 595 nm was continuously monitored. Similar experiments were performed using CS (Sigma-Aldrich); however, in this case, the experiments were performed in Tris-buffered saline (20 mM Tris, 150 mM NaCl, pH 8.0). Control experiments were performed using BSA (Sigma-Aldrich).

**Western Blot Analysis.** Proteins were subjected to electrophoresis as described above and transferred to nitrocellulose or PVDF membranes. After blocking overnight at 4 °C in skim milk solution (5% wt/vol) skim milk powder in PBS), the membranes were incubated with the relevant antibodies/streptavidin conjugates diluted in skim milk solution (1 h at 37 °C). Blots were imaged using a Typhoon Trio (GE Healthcare) or enhanced chemiluminescence. Additional details regarding the preparation of samples are provided in SI Experimental Procedures.

**Biotin-Streptavidin Pull-Down Assays.** Binding of α₂M to plasminogen-free FGN (Hyphen Biomed) and LDL (Source Bioscience) was assessed after biotinylation of the latter two species using (+)-biotin N-hydroxysuccinimide ester (Sigma-Aldrich). Binding of α₂M to Aβ₄₋₄₂ was performed using commercially prepared biotinylated Aβ₄₋₄₂ (bAβ₄₋₄₂; Cambridge Bioscience). Biotin-streptavidin pull-down assays were performed using Dynabeads My One Streptavidin C1 according to the manufacturer’s instructions (Life Technologies). Additional details are provided in SI Experimental Procedures.

**Th-T Assays.** Aβ₄₋₄₂ (5 μM; Cambridge Bioscience) was incubated at 28 °C with shaking in PBS containing Th-T (25 μM) in the presence or absence of native α₂M (200 nM) or oxidized α₂M (200 nM; pretreated using 20 or 50 μM NaOCl). The Th-T fluorescence of the samples was continuously monitored using a FLUOstar OPTIMA platereader (BMG Labtech Ltd.) with excitation and emission wavelengths of 440 nm and 480 nm (slit widths of 10 nm), respectively.

**Transmission Electron Microscopy.** Aβ₄₋₄₂ (5 μM) was incubated in the presence or absence of native or oxidized α₂M (250 nM) for 115 min at 28 °C with shaking. The samples were then applied to holey carbon-coated nickel grids (Agar Scientific) and imaged at the Multi-Imaging Centre in the Department of Physiology, Development and Neuroscience (University of Cambridge, Cambridge, UK), as previously described (13).

**Flow Cytometry.** The recombinant fusion protein RAP was purified as previously described (82). RAW 264.7, a murine leukemic monocyte/macrophage cell line, was donated by Claudia Monaco (University of Oxford, Oxford) and grown for 48 h without passage; then, it was detached using gentle scraping. The cells were then washed in Hank’s binding buffer (HBB) (composition in SI Experimental Procedures) by centrifugation and incubated with either 8 μM RAP in HBB or HBB alone (30 min at 4 °C). The cells were then washed again and incubated with α₂M or α₂M-client protein complexes (30 min at 4 °C) that were formed by incubating native or oxidized α₂M with native or oxidized bAβ₄₋₄₂ at RT. The binding of bAβ₄₋₄₂ was detected using fluorescent streptavidin conjugates. Additional experiments were performed, in which the cells were preincubated with native or oxidized α₂M and then washed before incubation with bAβ₄₋₄₂. Cell surface binding was measured using a BD FACSscan (Becton and Dickinson) at the Flow Cytometry Facility (Department of Pathology, University of Cambridge, United Kingdom). The data were analyzed using FlowJo7 software (Tree Star Inc.). The data shown are the composite geometric mean fluorescence of three independent samples containing 5,000–10,000 viable cells as determined by propidium iodide staining. The LR-D binding was calculated by subtracting the LR-I binding (i.e., the binding detected after pretreatment of the cells with RAP) from the total cell surface binding (i.e., the binding detected to cells not
incubated with RAP), and the errors were calculated using standard algorithms. Additional details are provided in SI Experimental Procedures.

**Cell Viability.** SH-SY5Y, a human neuroblastoma cell line, was seeded into a 96-well plate so that the cells reached ~80% confluence after 48 h. A\(\beta\)\_1–42 (233 \(\mu\)M) was incubated in PBS with gentle rotation (1 h at RT) followed by incubation in the presence or absence of \(\alpha_2\)M or oxidized \(\alpha_2\)M for another 30 min. The samples were then diluted to 17 \(\mu\)M A\(\beta\)_1–42 using Neurobasal media supplemented with B27 (Life Technologies), and this medium was applied to the cells. The cells were cultured for an additional 48 h before an 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed as previously described (18).

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**References**


Fig. 1. Effects of NaOCl on the structure and hydrophobicity of α2M. (A) Image of a native polyacrylamide gel showing purified α2M after treatment with 0–75 μM NaOCl overnight at RT. Also shown is the “fast” form of α2M generated by treatment with 400 mM NH4Cl, and α2M after dissociation into dimers using 8 M urea. B and C are images of Western blots using monoclonal anti-α2M antibody (clone 2N1/10) and anti-mouse IgG-horseradish peroxidase to detect α2M in human blood plasma after native polyacrylamide gel electrophoresis. In B, undiluted plasma was incubated with 0–2,000 μM NaOCl for 2 h at 37 °C before analysis. In C, undiluted plasma or plasma diluted 1:4 or 1:100 in PBS was incubated with 30 μM NaOCl for 2 h at 37 °C before analysis. (D) CD spectra of α2M after treatment with 0–50 μM NaOCl. Each dataset shown is the mean of six scans. (E) ANS-associated fluorescence (Excitation = 350 nm, Emission = 475 nm) measurements for α2M after treatment with 0–75 μM NaOCl and (F) ANS-associated fluorescence measurements of α2M after incubation in PBS only (native tetramer) or incubation with 15 μM NaOCl followed by SEC separation of dimeric and tetrameric α2M species. The data points are means (n = 3; ± SD). *Significant increase in ANS-associated fluorescence of NaOCl-treated α2M compared with the native control (Student t test; P ≤ 0.01). All fluorescence measurements are shown in arbitrary fluorescence units (AFU).
Fig. 2. Effect of NaOCl on the chaperone activity of α2M as measured by turbidity assays. (A) CPK at 3 μM was incubated at 43 °C in PBS ± native α2M. The molar ratios of α2M:CPK used are shown; (B) CS at 1.5 μM was incubated at 43 °C in Tris-buffered saline (TBS) ± native α2M. The molar ratios of α2M:CS used are shown. In C and D, oxidized α2M was prepared by treatment with NaOCl in PBS overnight at RT at the micromolar concentrations indicated in the key (e.g., ox12.5 denotes α2M incubated with 12.5 μM NaOCl) followed by extensive dialysis using the relevant buffer to remove any unreacted oxidant from the samples before the experiment. (C) CPK at 6 μM was incubated at 43 °C in PBS ± native or oxidized α2M at an approximate molar ratio of α2M:CPK of 1:9. (D) CS at 3 μM was incubated at 43 °C in TBS ± native or oxidized α2M at an approximate molar ratio of α2M:CS of 1:5. In E and F, SEC-purified oxidized α2M tetramer and oxidized α2M dimer were prepared by incubating native α2M with 15 μM NaOCl, as described above, followed by separation using SEC. (E) CPK at 5 μM was incubated at 43 °C in PBS ± native α2M, SEC-purified oxidized α2M tetramer, or oxidized α2M dimer at an approximate molar ratio of α2M:CPK of 1:9. (F) CS at 3 μM was incubated at 43 °C in TBS ± native α2M, SEC-purified oxidized α2M tetramer, or oxidized α2M dimer at an approximate molar ratio of α2M:CS of 1:5. For simplicity, the molar concentrations of α2M reported are calculated based on the intact 720 kDa tetramer, regardless of its NaOCl-induced dissociation into dimers. In all panels, turbidity was monitored using absorbance at 595 nm. The data shown are measurements from individual samples that are representative of several different experiments. Relevant control experiments are in Fig. S1.
Fig. 3. Effect of NaOCl on the binding of $\alpha_2$M to FGN and LDL. Images of Western blots detecting $\alpha_2$M using goat anti-$\alpha_2$M antiserum and anti-goat IgG-Alexa Fluor 488 performed after heating of the samples under reducing conditions and denaturing gel electrophoresis. (A) $\alpha_2$M was incubated overnight at RT with bFGN or bLDL ± NaOCl in PBS at the concentrations indicated. The biotinylated protein and any bound $\alpha_2$M were recovered using biotin-streptavidin pull-down assays. Also shown are the results for incubation of the streptavidin-coated beads with $\alpha_2$M only (−ve control) and the migration of native and oxidized $\alpha_2$M after being heating with reducing agents (+ve control), which results in $\alpha_2$M bands of ∼360 kDa (cross-linked dimer), 180 kDa (monomer), and 120 kDa (heat-liberated fragment). (B) Oxidized (ox) $\alpha_2$M, ox bFGN, and ox bLDL were prepared by separately incubating the proteins with 50 $\mu$M NaOCl in PBS overnight at RT and then removing any unreacted oxidant by dialysis. Ox bFGN or ox bLDL was subsequently incubated with native or ox $\alpha_2$M (ox50) in PBS for 1 h at RT before the biotinylated protein and any bound $\alpha_2$M was recovered using biotin streptavidin pull-down assays. bFGN and bLDL were independently detected using streptavidin-Alexa Fluor 594 and are shown below the respective blots detecting the bound $\alpha_2$M. The images shown are representative of several different experiments. Additional details in SI Experimental Procedures.
Fig. 4. Effect of NaOCl on the chaperone activity of α2M as measured by Th-T assay and transmission electron microscopy (TEM). (A) Aβ1–42 (5 μM in PBS, 25 μM Th-T) was incubated at 28 °C with shaking ± native or oxidized α2M (incubated with 20 or 50 μM NaOCl in PBS overnight at RT followed by dialysis to remove any unreacted oxidant; ox20 and ox50, respectively) at an approximate molar ratio of α2M:Aβ1–42 of 1:25, and the Th-T fluorescence (Excitation = 440 nm, Emission = 480 nm) was continuously recorded. The data shown are from individual samples that are representative of several different experiments, and the fluorescence intensity is reported in arbitrary fluorescence units (AFUs). The point at which samples were taken for analysis by TEM is indicated by the light gray vertical dashed line. (B) TEM images of Aβ1–42 after ∼115 min incubation ± native or oxidized (ox) α2M as described in A. The sample containing Aβ1–42 shows a large number of mature fibrils compared with the shorter prefibrillar aggregates present in the sample containing Aβ1–42 and native α2M. Mature fibrils are not detected and prefibrillar aggregates are much less abundant in samples containing ox α2M. (Scale bar: 100 nm.)
Fig. 5. Effect of NaOCl on the binding of α2M to Aβ1–42. (A) Image of a Western blot detecting α2M bound to bAβ1–42 in a biotin-streptavidin pull-down assay. bAβ1–42 was incubated in PBS ± 12.5 μM NaOCl for 1 h at RT. Unreacted NaOCl was quenched by the addition of excess L-methionine before native or oxidized α2M (incubated with 12.5 or 25 μM NaOCl; ox12.5 and ox25 respectively) were also added to the samples at a molar ratio of α2M:Aβ1–42 of 1:10. The samples were further incubated in PBS for 30 min at RT before bAβ1–42 and any bound α2M were recovered using the biotin-streptavidin pull-down assay. After heating and reduction, the samples were subjected to denaturing gel electrophoresis and Western blot analysis. (Upper) The larger blot shows bands of ~360 kDa (cross-linked α2M dimer), 180 kDa (α2M monomer), and 120 kDa (heat-liberated α2M fragment). (Lower) The smaller blot shows the bAβ1–42 recovered in each sample. (B) Image of a Western blot detecting the migration of native or ox Aβ1–42 by native gel electrophoresis and Western blot analysis. (Upper) The larger blot shows bands of ~360 kDa (cross-linked α2M dimer), 180 kDa (α2M monomer), and 120 kDa (heat-liberated α2M fragment). (Lower) The smaller blot shows the bAβ1–42 recovered in each sample. (C) Image of a Western blot detecting the migration of bAβ1–42 by denaturing gel electrophoresis after incubation with 0–25 μM NaOCl for 1 h at RT.
Fig. 6. Effects of oxidized (ox) α2M on the binding of bAβ1–42 to macrophage lipoprotein receptors and Aβ1–42 induced neurotoxicity. A–D show results of flow cytometric analyses of the binding of native and ox bAβ1–42 to RAW 264.7 macrophages. The cells were either preincubated with RAP, which inhibits lipoprotein receptors, or untreated before incubation with bAβ1–42 (2.5 μM in HBB) at 4 °C. The LR-D binding and LR-I binding were determined as described in Experimental Procedures. The cell surface binding of bAβ1–42 was detected using streptavidin-Alexa Fluor 488. The results shown are the composite geometric mean Alexa Fluor 488 fluorescence of 5,000 viable cells (n = 3 ± SD) in arbitrary fluorescence units (AFUs) and adjusted for background fluorescence. (A) bAβ1–42 was incubated ± native or ox α2M (preincubated with 25 μM NaOCl; oxα25) at an approximate molar ratio of α2M:bAβ1–42 of 1:10 for 20 min at RT before incubation with the RAW 264.7 cells. (B) Cells were incubated with 500 nM native or ox α2M or in HBB alone (control) and washed before subsequently being incubated with bAβ1–42. (C) Cells were incubated with ox bAβ1–42 or ox α2M, prepared by incubation with 25 μM NaOCl for 1 h at RT followed by the addition of excess L-methionine. (D) Cells were incubated with ox bAβ1–42 prepared as described in C and then preincubated for 20 min at RT ± native or ox α2M at an approximate molar ratio of α2M:bAβ1–42 of 1:10. (E) Viability of SH-SY5Y neuroblastoma cells as assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. SH-SY5Y cells were cultured ± Aβ1–42 (17 μM in neurobasal media), which had previously been incubated for 1 h in PBS to generate soluble oligomers and then incubated ± native or ox α2M (pretreated using 25 μM NaOCl) at an approximate molar ratio of α2M:bAβ1–42 of 1:150 for 20 min at RT. After 48 h, the cell viability was measured using the MTT assay, and the results shown are the mean (n = 6; ± SD) absorbance at 595 nm (A595 nm). ^Significant increases in cell surface bAβ1–42 binding and v significant decreases in cell surface bAβ1–42 binding (Student t test; P ≤ 0.05 in both cases). *Significantly increased cell surface binding of ox bAβ1–42 compared to bAβ1–42 (Student t test; P ≤ 0.01). **Significantly increased cell viability compared with cells treated with Aβ1–42 alone (Student t test; P ≤ 0.01).
Supporting Information

SI Experimental Procedures
The general details of Western blot analysis, biotin-streptavidin pull-down assays, and flow cytometry are provided in the text; however, additional details regarding the preparation of samples in these experiments are outlined below. Unless otherwise stated, all chemicals were obtained from Sigma-Aldrich.

Western Blot Analysis.

Analysis of \( \alpha_2 \)-macroglobulin in human blood plasma. Human blood was obtained from healthy donors and supplemented with sodium heparin and EDTA-free Complete Protease Inhibitor Mixture (Roche). The plasma was isolated by centrifugation at 1,300 \( \times \) g for 5 min and further supplemented with 0–2,000 \( \mu \)M sodium hypochlorite (NaOCl) before being incubated at 37 °C for 2 h. A 2-μl aliquot from each sample was subjected to native gel electrophoresis and transferred to nitrocellulose membrane using an XCell II blot module (Life Technologies), and it was blocked using skim milk solution [5% (wt/vol) skim milk powder in phosphate buffered saline (PBS)]. \( \alpha_2 \)-Macroglobulin (\( \alpha_2 \)M) was detected by enhanced chemiluminescence after incubating the blot with monoclonal anti-\( \alpha_2 \)M antibody (clone 2N1/10; Thermo Scientific) diluted 1:2,000 in skim milk solution followed by anti-mouse IgG-horseradish peroxidase (HRP) antibody (Bio-Rad) diluted 1:1,000 in skim milk solution. In similar experiments, plasma was diluted 1:4 or 1:100 before incubation with NaOCl and Western blot analysis as described above.

Analysis of the formation of complexes between \( \alpha_2 \) M and biotinylated creatine phosphokinase. Oxidized \( \alpha_2 \)M was prepared by incubating \( \alpha_2 \)M (690 nM) with NaOCl (12.5 or 25 \( \mu \)M) in PBS (overnight at room temperature (RT)). Additionally, \( \alpha_2 \)M was incubated in PBS alone (native control). All samples were dialyzed extensively in PBS before the experiment to remove unreacted NaOCl. Biotinylated creatine phosphokinase (bCPK; 5 \( \mu \)M) was incubated in the presence or absence of native or oxidized \( \alpha_2 \)M (500 nM) in PBS (RT or 43 °C overnight). The following day, the samples were centrifuged to remove any insoluble material, and the protein remaining in the supernatant was separated by native gel electrophoresis, transferred to nitrocellulose membrane using an XCell II blot module, and blocked using skim milk solution. bCPK was detected after incubating the blot with streptavidin-Alexa Fluor 488 (Life Technologies) diluted 1:5,000, and \( \alpha_2 \)M was detected after incubating the blot with goat anti-\( \alpha_2 \)M antiserum (Nordic Immunology) diluted 1:1,000 followed by anti-goat IgG-Alexa Fluor 584 antibody (Life Technologies); all detection reagents were diluted in skim milk solution.

Analysis of the formation of complexes between \( \alpha_2 \)M and amyloid β-peptide. Amyloid β-peptide (\( \beta_1-42 \); 5 \( \mu \)M) was incubated in the presence of \( \alpha_2 \)M (500 or 250 nM) in PBS ± NaOCl (30 \( \mu \)M) for 1 h at RT. The samples were separated by native gel electrophoresis or denaturing gel electrophoresis and transferred to nitrocellulose membranes using an XCell II blot module. \( \beta_1-42 \) was detected using monoclonal WO2 anti-\( \beta_1-42 \) antibody (Merk Millipore) diluted 1:2,000 followed by anti-goat IgG-Alexa Fluor 488 antibody diluted 1:2,000; both antibodies were diluted in skim milk solution. In a separate experiment, oxidized \( \alpha_2 \)M was prepared by incubating \( \alpha_2 \)M (690 nM) with NaOCl (12.5–50 \( \mu \)M) in PBS (RT overnight). Additionally, \( \alpha_2 \)M was incubated in PBS alone (native control). All samples were dialyzed extensively in PBS before the experiment to remove unreacted NaOCl. Oxidized \( \beta_1-42 \) was prepared by incubation of \( \beta_1-42 \) (110 \( \mu \)M) with NaOCl (25 \( \mu \)M) in PBS (RT overnight). Unreacted NaOCl was removed by extensive dialysis of the samples in PBS. Additionally, \( \alpha_2 \)M was prepared by incubating \( \alpha_2 \)M (690 nM) with NaOCl (25 \( \mu \)M) before separation by denaturing gel electrophoresis and transfer to PVDF membrane using an iBlot system. \( \beta_1-42 \) and \( \alpha_2 \)M were detected as described above.

Analysis of the effects of NaOCl on \( \beta_1-42 \) self-assembly. \( \beta_1-42 \) (110 \( \mu \)M) was incubated with NaOCl (0–25 \( \mu \)M at RT for 1 h) before separation by denaturing gel electrophoresis and transfer to PVDF membrane using an iBlot system. \( \beta_1-42 \) was detected as described above.

Biotin-Streptavidin Pull-Down Assays. Assay for the binding of \( \alpha_2 \)M to heat-denatured bCPK. Oxidized \( \alpha_2 \)M was prepared by incubating \( \alpha_2 \)M (690 nM) with NaOCl (25 \( \mu \)M) in PBS (RT overnight). Unreacted NaOCl was removed by extensive dialysis of the sample in PBS. Additionally, \( \alpha_2 \)M was incubated in PBS alone (native control). bCPK (5 \( \mu \)M) was incubated with native \( \alpha_2 \)M (500 nM) or oxidized \( \alpha_2 \)M (500 nM) in PBS (RT or 43 °C overnight). The following day, bCPK and any bound \( \alpha_2 \)M was detected as described above.
recovered using Dynabeads My One Streptavidin C1 (Life Technologies) according to the manufacturer’s instructions.

Protein bound to the beads was eluted by heating the samples at 100 °C in SDS sample loading buffer containing reducing agent. The eluted protein was separated by denaturing gel electrophoresis and transferred to PVDF membrane using an iBlot system. α2M was detected using goat anti-α2M antiserum followed by anti-goat IgG-Alexa Fluor 488 antibody (as above).

**Assays for the binding of α2M to oxidized biotinylated fibrinogen and biotinylated LDL.** α2M (345 nM) was incubated (RT overnight) with biotinylated fibrinogen (bFGN; 370 nM) or biotinylated LDL (bLDL; 1 mg/mL) in PBS containing NaOCl (0–75 μM). Additionally, α2M (690 nM) was incubated (RT overnight) in PBS or PBS containing NaOCl (50 μM). Buffer exchange was performed on all of the samples using Zeba Spin desalting columns, which had been equilibrated in PBS. The samples were then incubated with Dynabeads My One Streptavidin C1, as described above, to recover the biotinylated proteins and any bound α2M. The recovered α2M was detected by Western blot analysis as described above. In a separate experiment, α2M (690 nM), bFGN (740 nM), or bLDL (2 mg/mL) was oxidized by incubation with NaOCl (50 μM) in PBS (RT overnight). Additionally, α2M was incubated in PBS alone (native control). Buffer exchange was performed on all of the samples using Zeba Spin desalting columns as described above. Native or oxidized α2M (345 nM) was incubated with oxidized bFGN (370 nM) or oxidized bLDL (1 mg/mL) in PBS (RT for 1 h) before the biotinylated proteins, and any bound α2M was recovered using Dynabeads My One Streptavidin C1. The recovered α2M was detected as described above. The recovered bFGN and bLDL were detected after incubating the membrane with streptavidin-Alexa Fluor 594.

**Assays for the binding of α2M to biotinylated Aβ1–42 and oxidized biotinylated Aβ1–42.** Oxidized biotinylated Aβ1–42 (bAβ1–42) was prepared by incubating the peptide (110 μM) with NaOCl (12.5 μM) in PBS (RT for 30 min); additionally, bAβ1–42 was incubated in PBS alone (native control). To quench the reaction excess, L-methionine was added. Native or oxidized Aβ1–42 (5 μM) was then incubated (RT for 30 min) with native α2M (500 nM) or oxidized α2M (500 nM; pretreated using NaOCl, 12.5 or 25 μM, at RT overnight) and extensively dialyzed using PBS. bAβ1–42 and any bound α2M were recovered using Dynabeads My One Streptavidin C1. The recovered α2M was detected as described above, and the recovered bAβ1–42 was detected after incubating the membrane with streptavidin-Alexa Fluor 594.

**Tissue Culture.** SH-SY5Y, a human neuroblastoma cell line, was obtained from the American Type Culture Collection, grown in DMEM:F12 media supplemented with 10% (vol/vol) FBS, and routinely passaged using trypsin:EDTA. RAW 264.7 cells, a murine leukemic monocyte/macrophage cell line, were donated by Claudia Monaco (University of Oxford, Oxford), grown in high glucose DMEM supplemented with 10% (vol/vol) FBS, and routinely passaged using gentle scraping. All cell culture reagents and media were obtained from GE Healthcare. Cultured cells were maintained in a 37 °C incubator with 5% (vol/vol) CO2.

**Flow Cytometry.** Analysis of the binding of α2M to macrophage lipoprotein receptors. Oxidized α2M was prepared by incubating α2M (690 nM) with NaOCl (12.5–50 μM) in PBS (RT overnight). Additionally, α2M was incubated in PBS alone (native control). All samples were dialyzed extensively in PBS before the experiment to remove unreacted NaOCl. RAW 264.7 macrophage cells were detached by gentle scraping and washed in Hank’s binding buffer [HBB; 137 mM NaCl, 5.4 mM KCl, 0.34 mM Na2HPO4, 0.44 mM KH2PO4, 2 mM CaCl2, 2 mM MgCl2, 0.2% (wt/vol) bovine serum albumin (BSA), pH 7.4] by centrifugation. Cells were incubated stepwise with the following reagents in HBB (all incubations were for 30 min at 4 °C and separated by centrifugal washing of the cells): (i) receptor-associated protein (RAP; 8 μM) or HBB alone, (ii) native or oxidized α2M (345 nM), (iii) goat anti-α2M antiserum (diluted 1:1,000), and finally, (iv) anti-goat IgG-Alexa Fluor 488 antibody (diluted 1:1,000). After the final incubation step, the cells were washed two times and resuspended in HBB containing propidium iodide (PI; 3 μM) immediately before analysis using a BD FACSscan.

**Analysis of the binding of bCPK to macrophage lipoprotein receptors.** Oxidized α2M was prepared by incubating α2M (690 nM) with NaOCl (25 μM) in PBS (RT overnight). Unreacted NaOCl was removed by extensive dialysis of the sample in PBS. Additionally, α2M was incubated in PBS alone (native control). bCPK (5 μM) was incubated in the presence or absence of native α2M (500 nM) or oxidized α2M (500 nM) in PBS (RT or 43 °C overnight). RAW 264.7 macrophage cells were prepared as described above. Cells were incubated stepwise with the following reagents in HBB (all incubations were for 30 min at 4 °C and separated by centrifugal washing of the cells): (i) RAP (8 μM) or HBB alone, (ii) heated bCPK or heated mixtures of bCPK and native or oxidized as described above (diluted 1:1), and finally, (iii) streptavidin-Alexa Fluor 488 (diluted 1:1,000). After the final incubation step, the cells were washed two times and resuspended in HBB containing PI (3 μM) immediately before analysis using a BD FACSscan.
**Analysis of the binding of bAβ1-42 to macrophage lipoprotein receptors.** Oxidized α2M was prepared by incubating α2M (690 nM) with NaOCl (25 μM) in PBS (RT overnight). Unreacted NaOCl was removed by extensive dialysis of the sample in PBS. Additionally, α2M was incubated in PBS alone (native control). Oxidized bAβ1-42 was prepared by incubating the peptide (110 μM) with NaOCl (25 μM) in PBS (RT for 30 min). Additionally, bAβ1-42 was incubated in PBS alone (native control). To quench the reaction, excess L-methionine was added to both Aβ1-42 samples. Native or oxidized bAβ1-42 (5 μM) was incubated with native or oxidized α2M (500 nM) in PBS (RT for 20 min). RAW 264.7 macrophage cells were prepared as described above. Cells were incubated stepwise with the following reagents in HBB (all incubations were for 30 min at 4 °C and separated by centrifugal washing of the cells): (i) RAP (8 μM) or HBB alone, (ii) native or oxidized bAβ1-42 or mixtures of bAβ1-42 and native or oxidized α2M as described above (diluted 1:1), and finally, (iii) streptavidin-Alexa Fluor 488 (diluted 1:1,000). Other similar experiments featured the following incubation steps: (i) native or oxidized α2M (250 nM) or HBB alone, (ii) bAβ1-42 (2.5 μM), and finally, (iii) streptavidin-Alexa Fluor 488 (diluted 1:1,000). After the final incubation step, the cells were washed two times and resuspended in HBB containing PI (3 μM) immediately before analysis using a BD FACScan.

Fig. S1. Control experiments for the comparison of the chaperone activity of native and oxidized α2M. (A) Image of a native gel showing native or oxidized (ox) α2M (treated with 25 μM NaOCl in PBS overnight at RT followed by dialysis to remove any unreacted oxidant; ox25) after incubation for 3 h at 43 °C or 30 min at 60 °C. Also shown is the migration of control α2M and ox α2M, which have not been heated. (B) Turbidity measurements for native α2M or ox α2M (treated with 12.5 μM NaOCl; ox12.5) during heating at 43 °C. In C and D, ox BSA was prepared by treatment with NaOCl in PBS overnight at RT at the micromolar concentrations indicated in the key (e.g., ox12.5 BSA denotes BSA incubated with 12.5 μM NaOCl) followed by extensive dialysis using the relevant buffer before the experiment. (C) Turbidity measurements for 6 μM CPK incubated at 43 °C in PBS ± native or ox BSA at an approximate molar ratio of BSA:CPK of 1:9. (D) Turbidity measurements for 3 μM citrate synthase (CS) incubated at 43 °C in Tris-buffered saline (TBS) ± native or ox BSA at an approximate molar ratio of BSA:CS of 1:5. In B–D, turbidity was monitored using absorbance at 595 nm, and the data shown are measurements from individual samples that are representative of several different experiments.
Fig. S2. Effect of sodium thiocyanate (NaSCN) on the chaperone activity of α2M as measured by turbidity assays. (A) Image of an 8% Tris·Borate native polyacrylamide gel showing native α2M and α2M after treatment with 1.5 M NaSCN in PBS for 2 h at RT. (B) Turbidity measurements for 6 μM CPK incubated at 43 °C in PBS ± native α2M or NaSCN-treated α2M (as described in A and extensively dialyzed using PBS before the experiment) at a molar ratio of α2M:CPK of 1:20.

Fig. S3. Effect of high concentrations of NaOCl on the structure of α2M as assessed by native polyacrylamide gel electrophoresis. Image of a native polyacrylamide gel showing α2M after overnight treatment with 0–500 μM NaOCl at RT.

Fig. S4. Effect of NaOCl-induced oxidation of α2M on its binding to native bFGN as assessed by ELISA. Oxidized (ox) α2M was prepared by incubation with 12.5 or 25 μM NaOCl in PBS overnight at RT followed by dialysis to remove any unreacted oxidant (ox12.5 and ox25, respectively). Native α2M or ox α2M was serially diluted in the wells of an ELISA plate and left overnight at 4 °C. All wells were washed and subsequently blocked using 5% (wt/vol) BSA in PBS overnight at 4 °C. After washing, 1 μM bFGN in PBS containing 1% BSA was added to all wells and incubated at 37 °C for 1 h. The wells were washed again and incubated with a streptavidin-HRP conjugate (Sigma-Aldrich) according to the manufacturer’s instructions. After a final wash, orthophenylenediamine (2.5 mg/mL in 50 mM citric acid, 100 mM Na2PO4, pH 5) was added to all wells, and the reaction was stopped by the addition of 0.5 M HCl. The absorbance at 490 nm was measured using a FLUOstar OPTIMA platereader. The results shown are the mean absorbance (n = 3; ± SD) and have been adjusted for the background absorbance of wells coated with BSA only and incubated with bFGN as described above.
Fig. S5. Effects of NaOCl on the binding of α2M to misfolded bCPK and the delivery of misfolded bCPK to macrophage lipoprotein receptors. (A) Images of Western blots performed after native gel electrophoresis. The samples analyzed are bCPK ± native or oxidized (ox) α2M (pretreated with 12.5 or 25 μM NaOCl; ox12.5 and ox25, respectively) in PBS incubated overnight at RT (gray) or 43 °C (black) at an approximate molar ratio of α2M:bCPK of 1:10. (Left) The main blot shows the position of bCPK as detected using streptavidin-Alexa Fluor 488. (Right) The smaller blot shows the corresponding position of ox α2M (pretreated with 25 μM NaOCl) after heating with bCPK at 43 °C and Western blot detection after native gel electrophoresis using goat anti-α2M antiserum and anti-goat IgG-Alexa Fluor 594. (B) Composite size exclusion chromatograms, obtained using a Superose 6 column, showing analyses of bCPK, ox α2M, and a mixture of bCPK and ox α2M analyzed after heating at 43 °C; ‡ and + indicate the relative abundance of bCPK in the corresponding fractions of the lowermost trace after dot blot analysis using streptavidin-Alexa Fluor 488. The positions of molecular mass standards are shown, and the exclusion volume (V0) corresponds to ≥ 4 × 10^4 kDa. (C) Image of a Western blot performed after denaturing gel electrophoresis under reducing conditions. The samples are biotin-streptavidin immunoprecipitations of mixtures of bCPK incubated with native or ox α2M (pretreated with 25 μM NaOCl) in PBS incubated overnight at RT (gray) or 43 °C (black) at an approximate molar ratio of α2M:bCPK of 1:10. α2M coprecipitating with bCPK was detected using goat anti-α2M antiserum and anti-goat IgG Alexa Fluor 488. The visible bands correspond to 360 kDa (cross-linked α2M dimer), 180 kDa (α2M monomer), and 120 kDa (heat-liberated α2M fragment). (D) Flow cytometric analysis of the binding of bCPK to the surface of RAW 264.7 macrophages at 4 °C. RAW 264.7 macrophages were either preincubated with RAP, which inhibits lipoprotein receptors, or untreated before incubation with bCPK, which had been heated overnight at 43 °C ± native or ox α2M at an approximate molar ratio of α2M:bCPK of 1:10. The relative cell surface binding of bCPK (3 μM in HBB) was detected by incubating the cells with streptavidin-Alexa Fluor 488 followed by flow cytometric analysis. The results shown are the composite geometric mean Alexa Fluor 488 fluorescence of 5,000 viable cells (n = 3; ± SD) in arbitrary fluorescence units (AFUs) and adjusted for background fluorescence. The lipoprotein receptor-dependent (LR-D) binding and lipoprotein receptor-independent (LR-I) binding were determined as described in Experimental Procedures. ^Significant increases in the cell surface binding of bCPK as a result of heating bCPK in the presence of ox α2M (Student t test; P ≤ 0.01).
Fig. S6. Effect of NaOCl on the binding of α2M to macrophage lipoprotein receptors on RAW 264.7 cells as assessed by flow cytometry. Oxidized α2M was prepared by incubation with NaOCl overnight at RT at the concentrations indicated followed by dialysis to remove any unreacted oxidant. RAW 264.7 macrophages were either preincubated with RAP, which inhibits lipoprotein receptors, or untreated before incubation with native or oxidized α2M (345 nM in HBB). The cells were then incubated with goat anti-α2M antiserum (1:1,000) followed by anti-goat IgG-Alexa Fluor 488 antibody (1:1,000), both diluted in HBB. All incubation steps were carried out for 30 min at 4 °C and followed by washing of the cells by centrifugation. The results shown are the composite geometric mean Alexa Fluor 488 fluorescence of 5,000 viable cells (n = 3; ± SD) in AFUs and adjusted for background fluorescence. The LR-D binding and LR-I binding were determined as described in Experimental Procedures. ^Significant increases in the cell surface binding of α2M as a result of NaOCl treatment (Student t test; P ≤ 0.05).

Fig. S7. Western blot analysis of Aβ1–42 stock preparation. Image of a Western blot using W02 monoclonal anti-Aβ1–42 antibody and anti-mouse IgG-Alexa 488 secondary antibody to detect Aβ1–42; 1 or 0.1 μg native Aβ1–42 was subjected to denaturing gel electrophoresis followed by Western blot analysis. A small fraction of SDS-resistant oligomeric species was detected in the untreated stock solution; however, the majority of the peptide was monomeric.

Fig. S8. Viability of SH-SY5Y neuroblastoma cells as accessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. SH-SY5Y cells were cultured ± Aβ1–42 (17 μM in neurobasal media), which had previously been incubated for 1 h in PBS to generate soluble oligomers, and then incubated ± native or oxidized (ox) α2M (pretreated using 25 μM NaOCl; ox25) at an approximate molar ratio of α2M:bAβ1–42 of 1:200 for 20 min at RT. After 48 h, the cell viability was measured using MTT assay, and the results shown are the mean (n = 6; ± SD) absorbance at 595 nm (A595 nm). Asterisks denote significantly increased cell viability compared with cells treated with Aβ1–42 alone (Student t test; *P ≤ 0.05 and **P ≤ 0.01, respectively).
Fig. S9. Effect of NaOCl on the formation of dityrosine cross-links in oxidized $\alpha_2$M preparations. Dityrosine fluorescence (Excitation = 320 nm, Emission = 415 nm) measurements of $\alpha_2$M after treatment with 0–75 $\mu$M NaOCl. The data points are means ($n = 3; \pm SD$).

Fig. S10. Effect of NaOCl on the dissociation of $\alpha_2$M into dimers after 5 min at RT. Image of a native polyacrylamide gel showing $\alpha_2$M after incubation with 0–100 $\mu$M NaOCl for 5 min at RT.