Heme oxygenase-1 derived carbon monoxide suppresses Aβ1–42 toxicity in astrocytes

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Neurodegeneration in Alzheimer’s disease (AD) is extensively studied, and the involvement of astrocytes and other cell types in this process has been described. However, the responses of astrocytes themselves to amyloid β peptides (Aβ; the widely accepted major toxic factor in AD) is less well understood. Here, we show that Aβ(1-42) is toxic to primary cultures of astrocytes. Toxicity does not involve disruption of astrocyte Ca2+ homeostasis, but instead occurs via formation of the toxic reactive species, peroxynitrite. Thus, Aβ(1-42) raises peroxynitrite levels in astrocytes, and Aβ(1-42) toxicity can be inhibited by antioxidants, or by inhibition of nitric oxide (NO) formation (reactive oxygen species (ROS) and NO combine to form peroxynitrite), or by a scavenger of peroxynitrite. Increased ROS levels observed following Aβ(1-42) application were derived from NADPH oxidase. Induction of haem oxygenase-1 (HO-1) protected astrocytes from Aβ(1-42) toxicity, and this protective effect was mimicked by application of the carbon monoxide (CO) releasing molecule CORM-2, suggesting HO-1 protection was attributable to its formation of CO. CO suppressed the rise of NADPH oxidase-derived ROS caused by Aβ(1-42). Under hypoxic conditions (0.5% O2, 48 h) HO-1 was induced in astrocytes and Aβ(1-42) toxicity was significantly reduced, an effect which was reversed by the specific HO-1 inhibitor, QC-15. Our data suggest that Aβ(1-42) is toxic to astrocytes, but that induction of HO-1 affords protection against this toxicity due to formation of CO. HO-1 induction, or CO donors, would appear to present attractive possible approaches to provide protection of both neuronal and non-neuronal cell types from the degenerative effects of AD in the central nervous system.

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The progression of Alzheimer’s disease (AD), from early loss of functional synapses1–3 to the loss of neurones through apoptosis and other pathways4–7 has been described extensively, yet remains to be fully understood. The association of disease progression with increased levels of amyloid β peptide (Aβ; predominantly the 1–42 form, Aβ1–42) is also well established: this peptide is neurotoxic, and is also an important constituent of disease-characterising plaques.8,9

Astrocytes are as numerous as neurons in the central nervous system10 and their role in neurodegenerative diseases has also been extensively explored.11–13 These cells are diverse in structure and function, regulating CNS homeostasis in general and shaping activity at individual synapses.11,14,15 More recently there is a growing appreciation of the heterogeneity of astrocytes.16 In AD, and transgenic murine models of AD, atrophy of astrocytes is widely reported, and reactive astrocytes (in part defined as staining positively for glial fibrillar acidic protein; GFAP) have long been anatomically associated with plaques (reviewed in ref. 11). Atrophic loss of morphology clearly undermines the ability of astrocytes to regulate synaptic activity, and disrupts the ’neurovascular unit’ in which astrocytes play a central role in balancing local blood flow to neuronal activity.17,18

Numerous in vitro studies have also established that astrocytes can, at least in part, mediate the neurotoxic effects of Aβ. Thus, for example, extrasynaptic glutamate release from astrocytes in response to Aβ exposure leads to synaptic damage and loss.19 Evidence exists that suggests Aβ disrupts astrocyte [Ca2+]i, and by doing so activates reactive oxygen species (ROS) production by NADPH oxidase (reviewed in ref. 20). This increases lipid peroxidation in both astrocytes and neurones, which in turn depletes glutathione (GSH) levels in both cell types. Since neurones require delivery of GSH precursors from astrocytes, they are preferentially susceptible to continued oxidative stress and so die, whereas astrocytes have a greater antioxidant capacity to survive. It should be noted, however, that this model is not universally accepted, and others have indicated that astrocytic Ca2+ signalling is not disrupted by Aβ, at least over the same timecourse, and that different downstream signalling pathways are evoked.21,22

Despite the extensive literature on the involvement of astrocytes in the progression of neurodegeneration in AD, information available concerning the molecular mechanisms underlying responses of astrocytes themselves to Aβ is relatively limited and appears seemingly contradictory in some respects. Thus, for example, astrocyte viability has been reported to be unaffected or even slightly potentiated following exposure to sub-micromolar Aβ1–42 for 24 h.23 By contrast, others have shown that astrocytes are susceptible to micromolar Aβ – induced death over similar time periods.24 In the present study, we demonstrate that cortical astrocytes can undergo apoptotic death when exposed to sub-micromolar levels of Aβ1–42, and that this occurs via formation of peroxynitrite (ONOO−). Furthermore, we demonstrate a
complex pattern of modulation of this process of Aβ toxicity by the induction of the antioxidant enzyme haem oxygenase-1 (HO-1).

Results
Exposure of astrocytes to Aβ1-42 (10 nM-1 μM) for 24 h caused a concentration-dependent loss of viability, as shown in Figure 1a. This toxic effect of Aβ appeared selective, since the reverse sequence peptide, Aβ42-1, was without effect over the same concentration range (Figure 1a). In the presence of the caspase-3 inhibitor Z-DEVD-FMK (10 μM) the toxic effects of Aβ1-42 were partially reversed, suggesting the toxicity of Aβ1-42 was at least partly due to the induction of apoptosis.

Since it has previously been suggested that amyloid peptides disrupt [Ca2+]i in astrocytes, we next examined whether [Ca2+]i was altered in astrocytes at the levels we found to be toxic. As shown by the examples in Figure 2a, and the mean data of Figure 2b, exposure of astrocytes to 100 nM or 500 nM Aβ1-42 for 24 h caused a modest but significant reduction in resting [Ca2+]i whereas 500 nM reverse peptide (Aβ42-1) was without significant effect. Removal of extracellular Ca2+ (replaced with 1 mM EGTA) caused a reversible fall of [Ca2+]i in all cell groups examined, and basal [Ca2+]i under these conditions were similar across the 4 groups (Figure 2b, middle). Restoration of extracellular Ca2+ caused a transient overshoot of [Ca2+]i in control and reverse-peptide treated astrocytes, and this was significantly suppressed in cells exposed to Aβ1-42. This difference aside, our results are not consistent with the idea that Aβ1-42 is toxic due to its ability to raise [Ca2+]i as has previously been suggested.

ROS generation has often been associated with amyloid toxicity.25,26 To investigate any potential role for ROS in amyloid-mediated loss of astrocyte viability, we first examined the effects of MnTMPyP, a superoxide dismutase mimetic. As shown in Figure 3a, MnTMPyP significantly ameliorated the toxic effects of Aβ1-42. However, treatment of cells with mitochondria-targeted antioxidant TEMPO, a mitochondria targeted antioxidant, was unable to prevent Aβ1-42 toxicity.27 Figure 3b illustrates two images of APF fluorescence, before and during exposure to SNAP together with pyrogallol. Mean data are plotted in Figure 4c, which also illustrates the ability of ONOO− scavenger FeTPPs (5,10,15,20-tetrakis-[4-sulfonato-phenyl]-porphyrinato-iron[III]; 50 μM), which converts ONOO− rapidly to nitrate,31 to reduce the SNAP / pyrogallol rise of [Ca2+]i (100 μM), which auto-oxidises to form superoxide, a clear rise of APF fluorescence was apparent (Figures 4a–c). Figure 4b illustrates two images of APF fluorescence, before and during exposure to SNAP alone (Figure 4d).

The data in Figure 4 show clearly that formation of ONOO− in astrocytes is detectable, and is highly toxic. To investigate whether Aβ1-42 can exert its toxic actions through the formation of ONOO− we first examined its ability to increase APF fluorescence. As exemplified in the images of Figure 5a and the mean data of Figure 5b, APF fluorescence was indeed increased following a 24 h exposure to Aβ1-42. Consistent with

![Figure 1](image-url)
**Figure 2**  
$\alpha$-42 does not dramatically alter $[Ca^{2+}]$ in astrocytes. (a) Example microfluorimetric measurements of $[Ca^{2+}]$ in astrocytes under control conditions, or exposed to $\alpha$1-42 or $\alpha$42-1 as indicated, for 24 h. Scale bars apply to all traces. In each case, for the period indicated by the grey area, extracellular Ca$^{2+}$ was replaced with 1 mM EGT A. (b) Mean ± S.E.M. levels of $[Ca^{2+}]$ measured in 8–9 recordings under normal conditions (left), during exposure to Ca$^{2+}$-free solution (containing 1 mM EGT A; middle) and following replacement of Ca$^{2+}$ in the perfusate (right). Significance: * $P<0.05$; ** $P<0.01$; *** $P<0.001$ as compared with controls.

**Figure 3** Evidence for the involvement of NADPH oxidase-derived ROS in $\alpha$1-42 toxicity. (a) Effect on cell viability of a 24 h exposure of astrocytes to $\alpha$1-42 alone (10–500 nM, white bars) or $\alpha$1-42 in the additional presence of 100 $\mu$M MnTMPyP (grey bars). Bars represent the mean ± S.E.M. data of cells from 3 repeats (each performed in duplicate) with cells from different passages. (b) as (a), except cells were either treated with $\alpha$1-42 alone (10–500 nM, white bars) or $\alpha$1-42 in the additional presence of 10 $\mu$M Mito-TEMPO (grey bars). Bars represent the mean ± S.E.M. data of cells from 3 repeats (each performed in duplicate). Significance: *** $P<0.001$ as compared with respective controls, or between cells without drug versus MnTMPyP (a). NS, not significant. (c) Effect on cell viability of a 24 h exposure of astrocytes to $\alpha$1-42 alone (100–1000 nM, white bars) or $\alpha$1-42 in the additional presence of 1 mM apocyanin (grey bars). Bars represent the mean ± S.E.M. data of cells from 3 repeats (each performed in duplicate) with cells from different preparations. (d) as (c), except cells were either treated with $\alpha$1-42 alone (100–1000 nM, white bars) or $\alpha$1-42 in the additional presence of 10 $\mu$M GKT137831 (grey bars). Bars represent the mean ± S.E.M. data of cells from 2 repeats (each performed in duplicate) with cells from different preparations. Significance: ** $P<0.01$; *** $P<0.001$ as compared either with respective controls, or between drug treatment or no treatment, as indicated.
the idea that ONOO\textsuperscript{−} may contribute to its toxicity, we also found that A\textsubscript{β\textsubscript{1-42}} induced loss of astrocyte viability was essentially completely prevented in the presence of L-NAME to prevent NO formation (Figure 5c). Similarly, in the presence of the ONOO\textsuperscript{−} scavenger FeTPPS, A\textsubscript{β\textsubscript{1-42}} was without significant effect on astrocyte viability (Figure 5d). Together, these findings suggest that A\textsubscript{β\textsubscript{1-42}} is deleterious to astrocytes due to stimulation of elevated levels of both ROS and NO, which subsequently form ONOO\textsuperscript{−}.

We have previously shown that induction of HO-1 affords protection in neurons against the toxicity of A\textsubscript{β\textsubscript{1-42}}.\textsuperscript{32} To investigate whether HO-1 was similarly protective in astrocytes, we first induced its expression by exposing astrocytes to an HO-1 inducer, cobalt protoporphyrin IX (CoPPIX; 3 \(\mu\)M) for 24 h. As shown in Figure 6a such treatment caused a strong induction of HO-1, and in CoPPIX-treated astrocytes, the toxic effects of A\textsubscript{β\textsubscript{1-42}} (added for the same 24 h exposure to CoPPIX) were significantly attenuated (Figure 6b). Earlier studies have shown that carbon monoxide (CO), a product of HO-1-mediated haem degradation, can provide protection against apoptosis\textsuperscript{33,34} and so we investigated such a role for CO in astrocytes. As illustrated in Figure 6c, exposure of cells to the CO donor CORM-2 (3–20 \(\mu\)M) caused a concentration-dependent reversal of A\textsubscript{β\textsubscript{1-42}} toxicity without significantly affecting the viability of astrocytes not exposed to A\textsubscript{β\textsubscript{1-42}}. The control compound iCORM (which does not release CO) was unable to affect the toxicity of A\textsubscript{β\textsubscript{1-42}}. Neither CORM-2 nor iCORM induced significant levels of HO-1 themselves (Supplementary Figure 1). Similarly, L-NAME did not alter HO-1 expression significantly (Supplementary Figure 1). Biliverdin, another HO-1 product, was without effect on the toxicity of A\textsubscript{β\textsubscript{1-42}} (Supplementary Figure 2).

A previous study has reported that CO inhibits NADPH oxidase in proliferating smooth muscle.\textsuperscript{35} Since NADPH oxidase was a significant source of ROS mediating A\textsubscript{β\textsubscript{1-42}} toxicity (Figure 3), we investigated whether CO inhibition of NADPH oxidase activity accounted for its protective effects against A\textsubscript{β\textsubscript{1-42}} toxicity. To do this, we examined ROS formation...
using CellROX deep Red, a fluoroprobe which emits fluorescence upon oxidation. Representative images are shown in Figure 7a, and quantified in Figure 7b. As compared with untreated cells, those exposed to \( \text{A}^\beta_{1-42} \) showed a significant increase in ROS production (increased cytoplasmic fluorescence) and this was significantly reduced by the NOX1/NOX4 inhibitor, GKT137831 (10 \( \mu \)M). Fluorescence was also significantly suppressed by 20 \( \mu \)M CORM-2 but not by iCORM. Interestingly, CORM-2 alone caused a modest rise in fluorescence, presumably because it is known to stimulate ROS formation from mitochondria.\(^{36}\)

HO-1 is induced by several forms of cellular stress, prominent amongst which is hypoxia.\(^{37,38}\) We maintained astrocytes in hypoxia (0.5% \( \text{O}_2 \)) for 48 h and found this induced HO-1 strongly, as observed using both immunohistochemistry (Figure 8a) and western blotting (Figure 8b). Astrocytes maintained in hypoxia for a subsequent 24 h exposure to \( \text{A}^\beta_{1-42} \) were significantly more resistant to toxicity than those maintained under control (normoxic) conditions (Figure 8c). Hypoxic resistance to \( \text{A}^\beta_{1-42} \) toxicity appeared to be due specifically to HO-1 induction, since it was largely prevented by application of the selective HO-1 inhibitor QC-1539 (Figure 8c).

**Discussion**

The present study demonstrates that sub-micromolar levels of \( \text{A}^\beta_{1-42} \) have a significant impact on astrocyte viability over a 24 h period in comparison to a control peptide. Whilst these findings at least superficially agree with some previous studies,\(^{24}\) others have indicated that \( \text{A}^\beta_{1-42} \) does not alter astrocyte viability\(^{23}\) but can disrupt \([\text{Ca}^{2+}]_i\). These latter findings contrast with our observations both on viability (Figure 1) and alterations in \([\text{Ca}^{2+}]_i\) (Figure 2). At present we
cannot account for such different responses, but one likely possibility is the peptide preparation used. Our studies have employed peptides that were maintained for 24 h at 37 °C in medium before being applied to cells. We have previously shown that under these conditions cells are exposed to a mixture of monomers, small globular assemblies and protofibrils, as assessed by electron microscopy.32 By contrast, elevations in astrocytic [Ca2+]i were observed following acute application of 10–50 μM Aβ1–42 which was prepared in ultrapure water, reducing the likelihood of aggregation.40,41 Despite these reported differences, what is clear from this study and others is that Aβ1–42 can elevate astrocytic ROS levels. This is likely to be pathologically important, and precedes amyloid-induced increases of ROS in neurons.42 ROS derived from mitochondria have been implicated in ageing and associated with Ca2+ mobilisation in astrocytes.43 In the present study, NADPH oxidase(s) appear to be major contributors to the increased ROS levels (see ref. 44 and Figure 3). Although we have not explored the mechanism underlying amyloid-mediated stimulation of NADPH oxidase activity, it has previously been suggested that this occurs via Ca2+-dependent activation of protein kinase Cβ.44,45 Whether or not the same process underlies NADPH oxidase activation as reported here is unclear, but it is noteworthy that no elevation of [Ca2+]i was observed in the present study (Figure 2).

Astrocytes respond to pro-inflammatory signals with the release of toxic species, including ONOO− (see refs 46,47). It has previously been reported that astrocytes are relatively resistant to ONOO− toxicity when applied exogenously or derived from iNOS.48,49 Indeed, although NO modulates mitochondrial function in astrocytes, no overt toxicity has been reported50 in agreement with this study (Figure 4d). However, all three isoforms of nitric oxide synthase (NOS) are elevated in AD,51,52 and Lipton and colleagues have produced a number of studies which collectively provide compelling

Figure 6  HO-1 induction protects astrocytes from Aβ1–42 toxicity via CO formation. (a) Left, western blot for HO-1 taken from control astrocytes and astrocytes exposed to CoPPIX (3 μM) for 24 h, as indicated. β-Actin was also probed to confirm approximately equal protein loading of lanes. Below, mean ± S.E.M. (n = 3) relative densitometric readings for control and CoPPIX-treated cells, as indicated. *P < 0.05. Right, images of control and CoPPIX-treated cells, immunostained for HO-1. Scale bar applied to both images. (b) Effect on cell viability of a 24 h exposure of astrocytes to Aβ1–42 alone (100 nM, white bar) or Aβ1–42 in the additional presence of 3 μM CoPPIX. Also shown is the lack of effect of CoPPIX alone. Bars represent the mean ± S.E.M. data of cells from 5 repeats (each performed in duplicate) with cells from different preparations. ***P < 0.001. (c) Effect on cell viability of a 24 h exposure of astrocytes to 100 nM Aβ1–42 in the absence (white bar) or presence (dark grey bars) of the CO donor CORM-2 (3–20 μM). The effects of CORM-2 alone are also presented (light grey bars), along with the effects of 100 nM Aβ1–42 in the additional presence of the inactive form of CORM-2, iCORM (hatched bars). Bars represent the mean ± S.E.M. data of cells from 6 repeats (each performed in duplicate) with cells from different preparations. ***P < 0.001.
evidence that much of the toxicity of amyloid peptides arises due to stimulation of NO production. Aberrant, or excessive nitrosylation of target proteins (that is, conversion of cysteine –SH groups to –SNO groups) accounts for many of these deleterious effects. However, amyloid peptides have also been reported to increase ONOO\(^-\) levels \textit{in vivo}\textsuperscript{,46,47} and this can also impact on protein function through nitration of tyrosine residues.\textsuperscript{57} Indeed, A\(_\beta\)\textsubscript{1-42} has been reported to be a target of nitration, which can result in increased peptide aggregation.\textsuperscript{58} Given these reported differential sensitivities to NO and NO-related ROS, we examined the involvement of both NO and ONOO\(^-\). Consistent with previous studies our data highlighted that NO alone does not mediate amyloid toxicity. However, when added with a source of superoxide, pyrogallol, a dramatic rise of ONOO\(^-\) was observed, together with a large reduction in cell viability (Figure 4). These effects could be reversed by the ONOO\(^-\) scavenger, FeTPPs, the superoxide dismutase mimetic MnTMPyP, and also by L-NAME-mediated inhibition of NO formation (Figures 3, 4 and 5). As we also demonstrated that A\(_\beta\)\textsubscript{1-42} could directly raise ONOO\(^-\) levels (Figure 5b), our data strongly suggest that the toxic effects of A\(_\beta\)\textsubscript{1-42} on astrocytes are due to its ability to promote both ROS and NO formation and hence increase ONOO\(^-\) levels.

The cytotoxic actions of ONOO\(^-\) have been linked to intracellular glutathione levels and also to haem oxygenase activity.\textsuperscript{56} Induction of HO-1 in both neurons and astrocytes is well known to be associated with AD\textsuperscript{50,61} although whether this is beneficial, or contributes to disease progression, is subject to debate: HO-1 induction specifically in glia has been
shown to be detrimental because of the oxidative activity of iron liberated by haem degradation. However, we and others have provided evidence that HO-1 induction is protective. In the present study, HO-1 induction (either chemically or via exposure to hypoxia) is clearly protective against the toxic effects of Aβ1–42 and this protection is attributable to the formation of CO. Similarly in neurons we have shown that CO is protective against Aβ1–42 toxicity, but significantly the underlying mechanisms are quite distinct: in neurons, protection was attributable to inhibition of AMP-dependent protein kinase activation. In contrast, the present study demonstrates that CO provides protection via inhibition of ROS production in astrocytes specifically by NADPH oxidase. This correlates well with observations in smooth muscle cells and macrophages, where HO-1 induction, and resultant CO formation suppresses NADPH oxidase activity. This result is perhaps surprising, given that CO can itself increase ROS formation (Figure 7), not from NADPH oxidase but from mitochondria. Furthermore, CO can increase formation of NO in various cell types (e.g. refs 66,67) and can in some instances itself be damaging through formation of ONOO−, as shown in neuroblastoma (SH-SY5Y) cells. The source of ROS and / or the specific cell type is therefore likely to be key to outcomes. Clearly, the present study shows that CO is protective against Aβ1–42 toxicity in astrocytes, and this is mediated through suppression of NADPH oxidase activity.

In summary, we have shown that sub-micromolar concentrations Aβ1–42 are toxic to astrocytes, due to the activation of NADPH oxidase and subsequent elevation of ROS. In the presence of tonic NOS activity, ONOO− formation is increased. Induction of HO-1 provides protection against Aβ1–42 toxicity primarily via inhibition of NADPH oxidase. Our findings therefore further support the idea that HO-1 / CO is protective in the central nervous system and reveals potential mechanisms by which neuroprotection may be enhanced in the face of Aβ1–42 Cellular toxicity of AD.

Materials and Methods

Astrocyte preparation. To obtain primary cultures of astrocytes, cerebral cortices were removed from 5–7-day-old Wistar rats and placed in ice-cold phosphate-buffered solution (PBS) containing no Ca2+ or Mg2+ (Gibco, Thermo-Fisher, Paisley, UK). Meninges were removed and cortices were minced with a razor blade and dispersed into the same buffer containing 0.25 mg/ml trypsin, at 37 °C for 15 min. Trypsin digestion was halted by the addition of an equal volume of buffer supplemented with 16 μg/ml soy bean trypsin inhibitor (type I-S; Sigma Aldrich (Gillingham, UK), UK), 0.5 μg/ml DNase I (EC 3.1.21.1 type II from bovine pancreas; 125 kU/ml; Sigma Aldrich) and 0.3 mM MgSO4. The digested tissue was then pelleted by centrifugation at 400 x g for 5 min and the supernatant decanted before resuspending the cell pellet in 6.8 ml of buffer solution containing 100 μg/ml
soy bean trypsin inhibitor, 0.5 μg/ml DNase I and 1.5 mM MgSO4. Tissue was subsequently triturated gently with a 10 ml striptite (10 ×). The cloudy cell suspension was pipetted into 120 ml of media. The culture medium consisted of Eagle’s minimal essential medium supplemented with 10% foetal calf serum (v/v) and 1% (v/v) penicillin-streptomycin (Invitrogen, Paisley, UK). The cell suspension was then aliquoted into 75 cm2 flasks. Cells were then kept in a humidified incubator at 37 °C (95% air, 5% CO2). Six hours after plating out the cell suspension, cells were washed with fresh media to remove non-adhered cells and debris. This resulted in a culture of astrocytes (GFAP positive) as previously described.40 Culture medium was exchanged every 7 days and cells were grown in culture for up to 14 days.

**MTT assays.** Cell viability was investigated using MTT assays, as previously described.32 This technique compares well with the ATP-based CellTiter-Glo Luminescent Cell Viability Assay (Promega; SI Figure 3). Cells were cultured in poly-lysine coated 96-well plates to > 50% confluence or greater. Experiments were only carried out when all of the cell groups showed a similar confluency when viewed under the microscope. The final volume of each well after any treatment was kept at 100 μl. Cells were treated for 24 h with different concentrations of either Ap1–42 or the reverse peptide Aβ42-1, made up in serum-free media (SFM). The media in the control cells was also replaced with SFM for 24 h to ensure that all observations made were due to the application of Aβ rather than the result of serum withdrawal. This was done for all the experiments involving Aβ application.

When applying the tricarboxylicacidicorronenium(I) dimer (CORM-2) for 24 h, cells were treated twice a day (0930am and 1700 hours) to replenish the amount of CO in the media. Some cells were treated in parallel identically with CORM, the inactive, control compound which cannot release CO. Following the 24 h treatments with Aβ and CORM-2, the media was discarded and the cells washed gently with PBS. This step was repeated to get rid of all the CORM-2 as it reacts with the MTT. Then the PBS was replaced with 100 μl of fresh cell culture media in each well. For the MntMPxP experiments, the cells were pre-treated with MntMPxP for 30 min prior to applying Aβ1–42. For the L-NAM experiments, the cells were pre-treated with L-NAM for 1 h prior to treating with Aβ1–42. Next, 11 μl of Thiazoyl Blue Tetratolium Bromide (5 mg/ml, MTT, Sigma) made up in sterile PBS was added to each well (10% by volume) and the cells were incubated at 37 °C for 3 h. An equal volume (111 μl per well) of solubilizing solution consisting of isopropanol and HCl (24 ml propan-1-ol/ isopropanol/alcohol (Sigma) + 1 ml 1 M HCl) was added to each well to lyse the cells and the contents of each well was thoroughly mixed by pipetting. Absorbance was measured at 570 nm and at 630 nm using a spectrophotometer. The experiments were done in duplicate and repeated using cells from at least 3 different rat preparations to ensure the reliability of results. All of the results were normalised to untreated control cells and shown as a percentage change in cell viability compared to the corresponding controls.

**APF fluorescence.** APF (2-[[6-(4-amino) phenoxo-3H-xanthen-3-on-9-y]benzonic acid) fluorescence was used to detect peroxynitrite (ONOO−) formation as previously described.33 Cells were plated on to coverslips in 24-well plates and when needed for experiments coverslips containing cells were incubated with 100 nM, 500 nM, 1 μM Aβ1–42 or 500 nM Aβ42-1 for 24 h. Following the 24 h treatments the cells were incubated with 10 μM APF (Sigma Aldrich, UK) made up in HEPES buffered saline and incubated in the dark for 1 h at 37 °C. Following the 1 h incubation period, the coverslip was cut into fragments and one fragment was placed on a glass slide containing 200 μl of HEPES-buffered saline and incubated in the dark for 1 h at 37 °C. Following the 1 h incubation period, the coverslip was cut into fragments and one fragment was placed on a glass slide containing 200 μl of HEPES-buffered saline with 10 μM APF. APF was used due to its limited nonselective reactivity and resistance to light-induced auto-oxidation. Oxidation causes bright green fluorescence with an excitation/emission maxima of around 490/515 nm. Fluorescence increases when APF reacts with ONOO− and the changes in fluorescence intensity were measured using a ZEISS (Oberkochen, Germany) laser scanning confocal microscope (LSM 510). The change in fluorescence was measured continuously for a total of 10 min. The fluorophore was excited at 488 nm (emission was at 510 nm) by sequential scanning with argon lasers and the Zeiss AIM software was used to obtain the images. The same brightness, contrast and gamma settings were used for each condition.

**Western blotting.** Cells used for immunoblotting were cultured in T75 flasks and when confluent, were treated with Aβ1–42, Aβ42-1 or cobalt protoporphyrin (CoPPIX) at the concentrations indicated in the Results for 24 h. For the hypoxic experiments, cells were exposed to hypoxia (0.5% O2) for 48 h. Following the treatments, cells were washed in PBS and then lysed in lysis buffer containing complete protease inhibitor tablets (Roche) for 30 min at room temperature. Protein levels in the lysates were assessed using a BCA assay (Pierce). Cell proteins (typically 30 μg protein per lane) were separated on 12.5%, 0.75 mm thick polyacrylamide SDS gels and electrophoretically transferred onto 0.2 μm PVDF membranes (BioRad). The blots were blocked for 1 h with 10% milk protein in Tris-buffered saline with 0.05% Tween (TBST) then probed with primary antibody raised against HO-1 (1:200, rabbit polyclonal, Santa Cruz technologies or 1:1000 rabbit polyclonal, GeneTEX) at 4 °C overnight. Next, membranes were washed with TBST for 3 × 10 min prior to incubating with anti-rabbit horse radish peroxidase-conjugated secondary antibody (1:2000; Amersham Pharmacia Biotech, Buckinghamshire UK) for 1 h at room temperature. Following this incubation, membranes were washed in TBST for 3 × 10 min and bands visualised using an enhanced chemiluminescence detection system and hyperfilm ECL (Merck, UK).

**Immunofluorescence.** Cells were cultured on poly-lysine coated glass coverslips in 6-well plates at > 50% confluence prior to treatment with Aβ1–42, Aβ42-1 or cobalt protoporphyrin (CoPPIX) at the concentrations indicated in the Results for 24 h, prior to exposure to hypoxia (0.5% O2, 48 h). Following said treatments, cells were immunostained for HO-1 expression. Briefly, media was discarded and the cells were washed (3 × 5 min) with Dulbecco’s PBS. Cells were then fixed with paraformaldehyde (4% in PBS) for 20 min, following which they were permeabilized with PBS containing 0.22% Triton X100 supplemented with 10% normal goat serum (NGS; Sigma). Following 3 × 5 min washes with Dulbecco’s PBS containing 1% NGS, cells were then incubated overnight at 4 °C with the primary antibody; rabbit polyclonal anti-HO-1 (1:100, Santa Cruz) in Dulbecco’s PBS containing 1% NGS. The following day, cells were washed with Dulbecco’s PBS containing 1% NGS (3 × 5 min). Antibody binding was visualised by incubating the cells with a secondary antibody; Alexa Fluor-488 conjugated anti-rabbit IgG (1:1000, Invitrogen), for 1 h in the dark. Post-incubation, and following 3 × 5 min washes with Dulbecco’s PBS, coverslips were mounted on slides using Vectashield mounting media containing DAPI (Vector Laboratories, CA). The slides were then examined using a Zeiss laser scanning confocal microscope (LSM 700).

**Amylod beta preparation.** Aβ1–42 and Aβ42-1 (r-Peptides, Bogart USA) were dissolved in DMEM (Gibco, Paisley, UK) to make up 100 μM stock solutions and kept at −20 °C. In order to form protofibrils prior to treating the cells, the Aβ peptide was maintained at 37 °C for 24 h, as previously described.32

**CellROX assay.** Cells were cultured on poly-lysine coated glass coverslips in 6-well plates at > 50% confluence prior to treatment as described in the Results section. Following treatment the media was removed, cells were washed with PBS and 5 μM CellROX deep red regent (Molecular Probes, Life Technologies, Paisley, UK) was applied for 30 min in the dark at 37 °C. Thereafter, cells were washed three times with PBS and fixed with 10% buffered formaldehyde (Sigma) for 15 min. Cells were then washed with PBS and the coverslips were mounted on slides using Vectashield mounting media containing DAPI (Vector Laboratories, Burlingame, CA, USA). Slides were then examined using a Zeiss laser scanning confocal microscope (LSM 700). Images for all the treatments on a particular day were taken using identical settings. ImageJ software (NIH, Bethesda, USA) was used to analyse the images. To do this, 10 regions of interest were obtained for each image and 3–5 images each were taken for any given treatment on any given experimental day.

**Statistical analysis.** Data are shown as mean ± S.E.M. Statistical analysis was carried out using one-way ANOVA followed by either the Dunnett or Bonferroni post test, as appropriate. P-values of < 0.05 were considered significant. CellROX results were analysed using a two-way ANOVA followed by a Bonferroni post test. P < 0.05 was considered to be significant.

**Conflict of Interest**
The authors declare no conflict of interest.

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