Heme oxygenase-1 protects against Alzheimer’s amyloid-β1-42-induced toxicity via carbon monoxide production

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Heme oxygenase-1 (HO-1), an inducible enzyme up-regulated in Alzheimer’s disease, catabolises heme to biliverdin, Fe²⁺ and carbon monoxide (CO). CO can protect neurones from oxidative stress-induced apoptosis by inhibiting Kv2.1 channels, which mediates cellular K⁺ efflux as an early step in the apoptotic cascade. Since apoptosis contributes to the neuronal loss associated with amyloid β peptide (Aβ) toxicity in AD, we investigated the protective effects of HO-1 and CO against Aβ1-42 toxicity in SH-SY5Y cells, employing cells stably transfected with empty vector or expressing the cellular prion protein, PrPc, and rat primary hippocampal neurons. Aβ1-42 (containing protofibrils) caused a concentration-dependent decrease in cell viability, attributable at least in part to induction of apoptosis, with the PrPc-expressing cells showing greater susceptibility to Aβ1-42 toxicity. Pharmacological induction or genetic over-expression of HO-1 significantly ameliorated the effects of Aβ1-42. The CO-donor CORM-2 protected cells against Aβ1-42 toxicity in a concentration-dependent manner. Electrophysiological studies revealed no differences in the outward current pre- and post-Aβ1-42 treatment suggesting that K⁺ channel activity is unaffected in these cells. Instead, Aβ toxicity was reduced by the L-type Ca²⁺ channel blocker nifedipine, and by the CaMKII inhibitor, STO-609. Aβ also activated the downstream kinase, AMP-dependent protein kinase (AMPK). CO prevented this activation of AMPK. Our findings indicate that HO-1 protects against Aβ toxicity via production of CO. Protection does not arise from inhibition of apoptosis-associated K⁺ efflux, but rather by inhibition of AMPK activation, which has been recently implicated in the toxic effects of Aβ. These data provide a novel, beneficial effect of CO which adds to its growing potential as a therapeutic agent.

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Amongst the earliest of events leading to neuronal loss in Alzheimer’s disease (AD) is the loss of functional synapses,1–3 apparent long before deposition of amyloid β peptide (Aβ)-containing plaques.4 Although other parts of the neurone (e.g. the axon or soma) appear intact, their health at this early stage of disease progression is not clear. However, neurones ultimately die in AD and there is clear evidence that numerous events indicative of apoptosis occur even at early stages of disease progression.5–8 Thus, targeting of apoptotic mechanisms may be of therapeutic value in AD as well as in other neurodegenerative disorders. Furthermore, apoptosis is established as a mechanism of neuronal loss following other types of pathological stresses including ischemia associated with stroke,9 which can predispose individuals to the development of AD.10–12

Apoptosis is strongly influenced by intracellular K⁺ levels13 which regulate caspase activation, mitochondrial membrane potential and volume, osmolarity and cell volume.13,14 K⁺ loss via K⁺ channels is a key early stage in apoptosis,15–19 and K⁺ channel inhibitors can protect against apoptosis triggered by numerous insults including oxidative stress.20,21 Evidence suggests a particularly important role for the voltage-gated channel Kv2.1 in this process: expression of dominant negative Kv2.1 constructs (thus lacking functional Kv2.1 channels) protects against oxidant-induced apoptosis, and over-expression of Kv2.1 increases susceptibility to apoptosis.22,23 Pro-apoptotic agents cause a rapid increase in the surface expression of Kv2.1 channels,24 but whether or not this occurs in AD remains to be determined. Alternative pathways recently reported to promote cell death include activation of the AMP-dependent protein kinase (AMP kinase) which can act either as a Tau kinase25 or to inhibit the mTOR pathway26 and thus contribute to neurodegeneration.

Heme oxygenases (HO) are enzymes widely distributed throughout the body. In the central nervous system, HO-2 is constitutively expressed in neurones and astrocytes, while HO-1 is inducible in both cell types.27–30 Both HO-1 and HO-2

Abbreviations: Aβ, amyloid β peptide (Aβ); AD, Alzheimer’s disease (AD); AMPK, AMP-dependent protein kinase (AMPK); CaMKII, Ca²⁺/calmodulin kinase kinase II (); CORM-2, carbon monoxide releasing molecule; DMEM, Dulbecco’s minimal essential medium; DTPD, dithiopyridine (DTPD); HO-1, heme oxygenase-1 (HO-1); MTT, thiazolyl blue tetrazolium bromide; PI, propidium iodide; PBS, phosphate buffered saline

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break down heme to liberate biliverdin, ferrous iron (Fe$^{2+}$) and carbon monoxide (CO). This catalysis is of biological significance since it is crucial to iron and bile metabolism, and also generates a highly effective antioxidant in bilirubin (from biliverdin via bilirubin reductase). Numerous stimuli can induce HO-1 gene expression, including oxidative stress and $A_\beta$ peptides. Importantly, HO-1 is strikingly up-regulated in AD patients, a finding considered indicative of oxidative stress. Induction of HO-1 is clearly a neuroprotective response (although in some cases can exert detrimental effects). Our recent studies have demonstrated that CO protects against oxidant-induced apoptosis by selectively inhibiting Kv2.1. In the present study, we have investigated whether HO-1, or its product CO, can provide protection against $A_\beta$-induced toxicity in the human neuroblastoma, SH-SY5Y, and in rat primary hippocampal neurons, and whether this involves regulation of K$^+$ channels. We show that both HO-1 and CO protect cells against the toxicity of protofibrillar $A_\beta_{1-42}$ but that protection does not arise from inhibition of apoptosis-associated K$^+$ efflux, but rather by inhibition of AMPK activation.

**Results**

$A_\beta_{1-42}$-induced cell death in SH-SY5Y cells. To investigate any potential role of HO-1 and CO in affording protection against toxicity induced by $A_\beta_{1-42}$ (hereafter referred to simply as $A_\beta$), we first determined their effectiveness in SH-SY5Y cells either expressing the cellular prion protein (PrP$^C$) or containing the empty vector, as PrP$^C$ is a receptor for oligomeric and protofibrillar forms of $A_\beta$. Freshly dissolved $A_\beta$ which contained small globular structures (10 nm) and $A_\beta$ monomers (Figure 1a, upper images) had no effect on cell viability (not shown). After 24 h incubation at 37 °C, in addition to the small globular assemblies and monomers, the $A_\beta$ had formed protofibrils (25–90 nm in length) as assessed by electron microscopy (Figure 1a, lower images). These structures closely resembled the nanotubes that have recently been shown to mediate PrP$^C$-dependent and –independent synaptotoxicity. There was no evidence of any amyloid fibrils in our preparations. Using MTT assays to evaluate cell viability following exposure to $A_\beta$ for 24 h, we found that the protofibrillar $A_\beta$ caused a concentration-dependent loss of viability, and that cells over-expressing PrP$^C$ were significantly more sensitive to $A_\beta$ toxicity than the cells lacking PrP$^C$.

**Figure 1** Aggregation of $A_\beta_{1-42}$ into protofibrils is toxic to SH-SY5Y cells. (a) Representative images seen by negative stain electron microscopy of globular aggregates seen in $A_\beta$ freshly dissolved in DMEM (0 h, upper images). Boxed area of left-hand image is magnified in right-hand image to show more detail of these structures. Lower images show globular aggregates plus protofibrillar structures in $A_\beta$ solutions stored at 37 °C for 24 h. Boxed area of left-hand image is magnified in right-hand image to show more detail of these structures. Scale bar in all images 50 nm. (b) Effect of $A_\beta_{1-42}$ on cell viability in SH-SY5Y cells containing empty vector (white bars) and stably expressing PrP$^C$ (gray bars) using the mitochondrial activity-based MTT assay. Bars represent the mean ± S.E.M. data of cells from 10 repeats (each performed in duplicate) with cells from different passages. (c) The effect of different concentrations of $A_\beta_{1-42}$ (white bars) and the reverse sequence $A_\beta_{42-1}$ (gray bars) on cell viability in empty vector containing and PrP$^C$-expressing cells. Bars show the mean ± S.E.M. of four repeats. Statistical significance was determined with a one-way ANOVA followed by Bonferroni post-hoc test. **P < 0.01, ***P < 0.001. (d) Effect of oxidants on cell viability in empty vector containing (white bars) and PrP$^C$-expressing (gray bars) cells. Left, effect of different concentrations of DTDP (10 min exposure, left) on cell viability. Each bar represents the mean ± S.E.M. of seven repeats. Right, cell viability following a 30 min exposure to various concentrations of diamide. Each bar shows the mean ± S.E.M. of five repeats using cells from different passages. Statistical significance is denoted by **P < 0.01, compared to the corresponding control cells.
Aβ-induced cell death is at least partly attributable to apoptosis. To investigate whether Aβ-induced toxicity arose from induction of apoptosis, two separate approaches were taken. The reduction in cell viability caused by Aβ was significantly reduced by two distinct caspase inhibitors, the pan-caspase inhibitor (Q-VD-OPh; 1 μM) or the irreversible caspase inhibitor III (Boc-D-FMK; 10 μM) (Figure 2a), consistent with the idea that Aβ toxicity involves activation of apoptosis. In both PrPc-expressing cells and empty vector containing cells, Aβ caused an increase in the proportion of both CellEvent positive and PI-positive cells (i.e. cells showing increased caspase activity and loss of viability), approaching levels seen in cells treated with staurosporine, which induced positive caspase and PI staining in 80–100% of cells (Figure 2b). These data further support an important role for apoptosis induction in the Aβ-induced loss of cell viability. Using this same assay, we also observed that the CO-donor, CORM-2, significantly attenuated the number of CellEvent and PI-positive cells following exposure to 0.5 μM Aβ (Figure 2b), suggesting a possible protective role for CO against Aβ toxicity. Importantly, we confirmed this potentially protective effect of CO in cultured rat primary hippocampal neurones (Figure 2c). Thus, employing cultures ranging from 7–21 days in vitro, we found that the same protofibrillar preparation of Aβ at a concentration of 100 nM caused marked increases in the number of apoptotic cells (indicated by CellEvent positive cells). Effects were found at all culture ages, and Aβ was more potent in inducing apoptosis in hippocampal neurones than in the SH-SY5Y cells (Figure 2c). In the presence of CORM-2 (10 μM), the effects of Aβ on hippocampal neurones were largely reversed, and CORM-2 was without significant effect itself on apoptosis (Figure 2c). These findings indicate that the neuroprotective effects of CO against Aβ toxicity can be observed in different neuronal preparations.

![Figure 2](image-url)
HO-1 induction protects against Aβ toxicity. In order to investigate the ability of HO-1 to protect against Aβ toxicity, cells were exposed to two established inducers of this enzyme. Exposure to either 30 μM hemin or 3 μM CoPPIX for 24 h significantly attenuated the toxic effects of Aβ in both empty vector containing (Figure 3a) and PrPc-expressing SH-SY5Y cells (Figure 3b). HO-1 induction was verified by immunocytochemistry and western blotting in response to hemin (similar results obtained for CoPPIX; data not shown). Densitometric analysis indicated that hemin increased HO-1 expression to 188.0 ± 5.0% of control levels (i.e. 1.88-fold; \( P < 0.001, n = 4 \) repeats) in empty vector containing cells, and by 239.5 ± 28.8% (\( P < 0.05, n = 4 \) experiments) in PrPc-expressing cells. These data suggested that HO-1 affords protection against Aβ in addition to its known ability to protect against oxidant-induced apoptosis. To investigate this further, we over-expressed HO-1 in SH-SY5Y cells. As shown in Figure 3c, HO-1 over-expression also significantly diminished the toxic effects of Aβ. Since exposure to Aβ is clearly deleterious to cells, we also examined whether Aβ itself could induce expression of HO-1. As shown by western blotting in Figure 3d, 24 h exposure to Aβ did indeed increase expression of HO-1 in empty vector and PrPc-expressing cells, respectively. Interestingly, there was differential sensitivity to Aβ in the two cell groups: For empty vector cells, a graded increase in HO-1 induction was observed, but in PrPc-expressing SH-SY5Y cells significant induction was observed in response to 100 but not 500 nM Aβ. In both cell groups, induction was modest compared with the effects of hemin or CoPPIX.

Hypoxia has long been known to induce HO-1 expression, so we investigated the sensitivity to Aβ of cells which had been maintained in a hypoxic environment (0.5% O\(_2\), 24 h) prior to (and during) exposure to Aβ. Hypoxia did indeed induce HO-1 expression, as determined immunocytochemically, and hypoxic cells (both PrPc-expressing and empty vector containing cells) were significantly more resistant to the toxic actions of Aβ (Figures 4a and b). This was particularly prominent in the PrPc-expressing cells. We therefore used these cells exclusively to examine the ability of...
the HO-1 inhibitor QC-15\textsuperscript{43} to modulate the responses of hypoxia-exposed cells to Aβ. As shown in Figure 4c, QC-15 reversed the protective effects of hypoxia against Aβ toxicity, suggesting strongly that the protective effects of hypoxia were specifically because of its induction of HO-1.

CO protects against Aβ toxicity: lack of involvement of a K⁺ channel ‘surge’. In order to examine whether the protective effects of HO-1 induction / over-expression could be attributable to its ability to generate CO (as suggested by the data presented in Figure 2), we examined the ability of the CO-donor, CORM-2, to protect cells against Aβ toxicity. In both empty vector containing cells (Figure 5a) and PrP\textsuperscript{c}-expressing cells (Figure 5b), exposure of cells to CORM-2 caused a concentration-dependent reduction in the toxic effects of Aβ. The control compound, iCORM, was without significant effect, and neither CORM-2 nor iCORM affected viability when applied alone. These data support the hypothesis that HO-1 affords protection against Aβ toxicity via the production (and subsequent action) of CO. Whole-cell patch-clamp recordings revealed that K⁺ current densities, indicative of K⁺ channel activity at the plasma membrane, were not significantly affected by exposure to Aβ for 24 h in either the vector only containing cells (Figure 5d) or in the PrPc-expressing cells (Figure 5e). However, it was noted that K⁺ current density was significantly reduced in PrPc-expressing cells as compared with empty vector containing cells. The reasons for this are currently unknown. This notwithstanding, cell death induced by Aβ was not attributable to the K⁺ channel ‘surge’ associated with oxidant-induced apoptosis, indicating that HO-1/CO provided protection against Aβ toxicity via an alternative mechanism.

Involvement of the CaMKKII/AMPK pathway in Aβ neurotoxicity. Recent studies have suggested that Aβ neurotoxicity can involve activation of the Ca\textsuperscript{2+}/calmodulin kinase II (CaMKII)/AMPK pathway.\textsuperscript{25,26} To investigate the involvement of this pathway in Aβ-induced neurotoxicity in SH-SY5Y cells as reported here, we first investigated the effects of suppressing Ca\textsuperscript{2+} influx into cells using the L-type Ca\textsuperscript{2+} channel inhibitor nifedipine. Nifedipine significantly attenuated Aβ toxicity in both empty vector containing cells and PrPc-expressing cells (Figure 6a), suggesting Ca\textsuperscript{2+} influx via L-type Ca\textsuperscript{2+} channels is involved in Aβ toxicity. Since a rise of [Ca\textsuperscript{2+}] can lead to activation of CaMKII, we next explored the involvement of this kinase in Aβ toxicity, and found that exposure of cells to the CaMKII inhibitor STO-609 also significantly attenuated Aβ toxicity (Figure 6b). Exposure to both nifedipine and STO-609 produced no additional attenuation above that observed by
either agent applied alone (data not shown). In agreement with recent studies, we also found that Aβ activated AMPK, as demonstrated by a specific increase in the level of AMPK phosphorylation (without change in total AMPK expression levels (Figure 7)). In further support of this, we found that Aβ increased the level of ACC phosphorylation, the major AMPK substrate. Importantly, CO (applied as CORM-2) prevented AMPK activation and subsequent phosphorylation of ACC (Figures 7a and b).

Discussion

The present study aimed to investigate whether HO-1, via CO generation, provides neuroprotection against the toxicity of Aβ. There is compelling evidence to suggest that increased HO-1 expression can be neuroprotective against exposure to glutamate, oxidants or physical damage, and this protection may be attributable, at least in part, to the production of CO (e.g. Zeynalov et al.36). Although much of the cellular damage associated with AD is reminiscent of oxidative damage caused by such agents/interventions, it is important to establish the specific mechanisms underlying neurodegeneration in AD, in order to understand any protective actions of HO-1/CO and so, potentially, exploit such actions in the development of new anti-neurodegenerative therapies. Indeed, whilst expression of HO-1 has long been known to be increased in AD patients,27 it remains to be fully resolved whether this is beneficial or detrimental. Detrimental effects arising from increased HO-1 expression specifically in glia, have been associated with excessive deposition of iron as a consequence of increased heme degradation.46–48 However, other studies suggest increased astrocytic HO-1 expression can provide protection for nearby neurons arising specifically from the production of CO.49

The present study indicates that HO-1 is indeed protective, and protection appears to arise via the production of CO. Our study employed SH-SY5Y cells over-expressing PrPc and demonstrated that such cells were more vulnerable to the toxicity of Aβ when compared with the empty vector containing cells which lack endogenous PrPc expression50 (Figure 1). Aβ has been show to aggregate and assemble into a range of dynamic, soluble species, and defining the particular in vivo ‘toxic’ species which correlate most significantly with AD progression is challenging. PrPc has been identified as a high-affinity receptor for oligomeric Aβ.38,39,51–53 PrPc is thought to be a critical mediator of the synaptic loss, neurotoxicity, long-term potentiation impairments and memory deficits that are
caused by these Aβ oligomers. However, the heterogeneity and lack of characterization of the synthetic Aβ preparations used in some of these studies makes it difficult to define the active PrPc-dependent Aβ toxic assemblies. A recent report identified protofibrils to be a key PrPc-specific binding species, defining their triple helical structure as Aβ nanotubes. These particular structures of Aβ caused PrPc-dependent synaptotoxicity. Electron microscopy indicated that our preparation of Aβ resembles those used in that particular study, and thus provide an explanation as to why our PrPc-expressing cells were selectively more vulnerable to Aβ toxicity, when their sensitivity to apoptosis induced by oxidants was similar to that seen in cells lacking PrPc (Figure 1).

Several lines of evidence suggest that HO-1 provides neuroprotection, reducing the toxic effects of Aβ: thus, chemical induction of HO-1 with protoporphyrins (Figures 3a and b) or over-expression of HO-1 (Figure 3c) reduced the toxic actions of Aβ. Furthermore, protection was provided by hypoxic induction of HO-1 (Figure 4). Such hypoxic treatment can alter the expression of many proteins which might contribute to neuroprotection (see e.g. yet this effect of hypoxia was prevented by the selective HO-1 inhibitor, QC-15. Collectively, these data strongly suggest that HO-1 provides protection against the toxic actions of Aβ. Furthermore, our results also indicate that HO-1 is likely to be protective because of its ability to generate CO. Thus, although HO-1 activity was not assessed directly (e.g. via measurement of CO or bilirubin production) the established CO-donor, CORM-2 (but not the inactive form, iCORM) mimicked the effects of HO-1 induction (Figures 2 and 5) to provide protection against Aβ toxicity. Furthermore, another HO-1 product, biliverdin, was without protective effect (Figure 5).

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Our previous work has indicated that HO-1, through the production of CO, protects hippocampal neurons from oxidant-induced apoptosis by inhibiting the voltage-gated K+ channel Kv2.1. Similarly, HEK293 cells over-expressing Kv2.1 displayed increased vulnerability to oxidant-induced apoptosis, and this was also prevented by CO inhibition of Kv2.1. Since earlier reports had suggested that toxic effects of Aβ might arise due to its ability to increase outward K+ currents, we explored this as a possible mechanism by which CO might protect SH-SY5Y cells against Aβ toxicity. Perhaps surprisingly, K+ currents in SH-SY5Y cells were...
unaffected by the same levels of Aβ which induced toxicity (Figure 5). The discrepancy between this finding and earlier reports on the ability of Aβ to augment K⁺ currents is not clear at present, but it is noteworthy that previous studies employed Aβ at high (>$10$ μM) concentrations, whereas the present study largely employed sub-micromolar concentrations of Aβ. Our results indicate that K⁺ channel modulation is unlikely to provide significant relief from Aβ toxicity (Figure 6a). This finding prompted us to investigate potential Ca²⁺-dependent pathways associated with Aβ toxicity. Several studies have implicated AMPK - which can be activated by the Ca²⁺-dependent upstream kinase, CaMKKβ - as exerting important influences on the development of AD. Thus, for example, Thornton et al., demonstrated that exposure of cortical neurons to Aβ (20 μM, 30 min) activates AMPK in a CaMKKβ-dependent manner and, importantly, that AMPK subsequently acts as a Tau kinase, contributing to its hyperphosphorylation. Yoon et al. also reported that Aβ activates AMPK, and indicated that this led to endoplasmic reticulum stress arising from inhibition of the mTOR pathway. This in turn led to activation of JNK3 which, crucially, phosphorylated APP (amyloid precursor protein). This phosphorylation of APP promotes its internalization and cleavage to generate increased levels of Aβ. Most recently, Ca²⁺ and CaMKKβ-dependent AMPK activation by Aβ was confirmed in a study which also demonstrated that activation of the kinase led to a loss of dendritic spines (an early feature of AD) and also to Tau phosphorylation. Our data indicate that Aβ activates AMPK in SH-SYSY cells (Figure 7). Furthermore, this activation is prevented by CO. The exact site at which CO acts to prevent AMPK activation, and therefore provide neuroprotection, remains to be determined. However, this is worthy of further exploration: despite its deserved reputation as a potent toxin, the physiological actions of endogenous CO, and its potential as a therapeutic agent in numerous disorders, is increasingly being recognized (e.g. Motterlini et al.). Future studies will reveal whether it may also be of benefit in combatting neurodegenerative diseases.

Figure 7  CO prevents AMPK activation by Aβ. (a) Example western blots showing total and phosphorylated AMPKα, total and phosphorylated acetyl-CoA carboxylase (ACC) and the corresponding β-actin levels in lysates from PrPc-expressing cells. Cells were either untreated, or exposed to Aβ (100 and 500 nM, 24 h), or to 500 nM Aβ in the additional presence of 20 μM CORM-2, or to CORM-2 alone, as indicated. (b) Mean (± S.E.M., from three experiments) densitometry data under each of the conditions exemplified in (a). Statistical significance indicated by *P<0.05 when compared with corresponding control and corresponding Aβ-treated cells, respectively.

Materials and Methods

Tissue culture. All experiments were conducted using SH-SYSY cells stably transfected with either empty pIREsneo vector (BD Biosciences, Oxford, UK; termed empty vector containing cells in this study) or engineered to express murine PrP containing the 3F4 epitope tag (human M108/M111; termed PrP cells), as described previously. Cells were cultured in DMEM medium containing glutamine, supplemented with 10% (v/v) fetal calf serum, penicillin (100 U/ml) and streptomycin (100 U/ml) (all from GIBCO Life Sciences, Paisley, Scotland, UK). Cells were incubated at 37 °C in a humidified incubator gassed with 95% air and 5% CO₂, and passaged every 7 days and used up to 10 passages.

Human HO-1 over-expressing SH-SYSY cells were generated in-house and cultured using DMEM media with glutamine, supplemented with 10% (v/v) fetal calf serum, penicillin (100 U/ml), streptomycin (100 U/ml) and fungizone (all from Gibco). Cells were incubated at 37 °C in a humidified incubator gassed with 95% air and 5% CO₂, and passaged every 7 days and used up to 10 passages.

Cell Death and Disease
according to manufacturer's instructions. Stably transfected cell lines were selected with
hygromycin B antibiotic (200 μg/ml, Calbiochem) added 3 days after transfection.
Selection was applied for 4 weeks (media changed every 4-5 days), colonies were
then picked, grown to confluence and screened by western blotting for HO-1
expression after culturing in T25 flasks for 24–48 h. Hygromycin B selection
was maintained throughout the cloning process at 200 μg/ml and in all subsequent
passages once stable clones had been positively identified.

Primary cultures of hippocampal neurones. Hippocampi of 6–8-day-old
Wistar rats were removed as described previously. Brain tissue was
incubated with 0.25% (v/v) trypsin for 15 min at 37 °C in phosphate buffered saline
(PBS). Trypsin digestion was terminated by the addition of equal amounts of PBS,
supplemented with 16 μg/ml soybean trypsin inhibitor (type I-S; Sigma, Paisley, UK),
0.5 g/ml DNase I (type II from bovine pancreas; 125 kilounits/ml), Sigma), and
1.5 mM MgSO4. The tissue was then pelleted by centrifugation at 3000 × g for 5 min,
resuspended in 2 ml of PBS with 100 μg/ml soybean trypsin inhibitor, 0.5 μg/ml
globular assemblies and protofibrillar structures in each resulting image were
measured after setting the pixel/scale ratio.

EM images were analyzed
using FIJI (ImageJ-2). Briefly, all images were magnified (400%) using Photoshop
for 24 h to form oligomers prior to treating the cells. EM images were analyzed
μtreatment was kept at 100
for 24 h to ensure that any effects observed were due to A
Likewise, the media in control wells was also replaced with serum-free media for
24 h following 3 × 5 min washes with Dulbecco's PBS, coverslips were mounted on slides using Vectorshield® (Vector Laboratories, Peterborough, UK). The coverslips were sealed and examined using a Nikon E600 light microscope (Nikon, Kingston upon Thames, UK). All the images were obtained using the x40 lens and Q image microscopublisher ACQuis (Syncroscope, Cambridge, UK) software. At least three fields of interest were taken for each slide, and the number of cells with Hoechst positive nuclei that also stained
positive for CellEvent or PI were counted. The data is presented as a percentage of
(+ CellEvent or PI stained cells compared to the corresponding Hoechst (+) cells.
Hippocampal neurones were cultured on poly-lysine coated coverslips for 7, 14
or 21 days. On the specific days, the coverslips were washed twice with sterile PBS
twice to get rid of cell debris and the cells were then treated for 24 h with fresh
media for the control cells or media containing 100 nM Aβ1-42, 10 μM CORM or
100 nM Aβ1-42+10 μM CORM. Following the 24 h incubation period, the cells were
stained with the CellEvent dye and examined and analyzed exactly as for
SH-SY5Y cells.

Immunocytochemistry. Cells were cultured on poly-lysine coated glass
coverslips in 6-well plates at >50% confluence prior to treatment with either 3 μM
cobalt protoporphyrin (CoPPPIX) or 30 μM chloroferriprotoporphyrin (hemin;
Calbiochem) for 24 h, or prior to exposure to hypoxia (0.5% O2, 48 h). Following
said treatments, cells were immunostained for HO-1. Briefly, media was discarded and
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, Heidelberg, Germany)
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 visualized
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
Vektashield® mounting media containing DAPI (Vector Laboratories).
The slides were then examined using a Zeiss (Cambridge, UK) laser scanning
confocal microscope (LSM 510).

Electrophysiology. Fragments of coverslip with attached cells were
transferred to a continuously perfused (3–5 ml/min) recording chamber mounted
on the stage of an Olympus (Southend, UK) CK40 inverted microscope. All
experiments were carried out at 22 ± 1 °C, unless otherwise stated. Cells were
continually perfused with a solution containing (in mM): 135 NaCl, 5 KCl, 1.2 MgCl2,
2.5 CaCl2, 2Na₂ATP (pH 7.2 with KOH). Whole-cell patch-clamp
recordings were then obtained in voltage-clamp mode with cells clamped at
−70 mV. Patch pipettes had resistances 4–6 MΩ when filled with an intracellular
solution consisting of (in mM): 10 NaCl, 117 KCl, 2 MgCl2, 10 D-glucose (pH 7.4 with NaOH). Whole-cell patch-clamp
measurements were performed using the Digidata 1322A interface (Axon
Instruments Inc.). Data were filtered at 1 kHz and digitized at 2 kHz. To evoke ionic
outward K+ currents in SHSY5Y cells, a series of 100 ms depolarizing steps from
−90 to +60 mV in 10 mV increments, were employed. Offline analysis was carried
out using the data analysis package Clampfit 9 (Axon Instruments) and data are
expressed as mean ± S.E.M.

Western blotting. Cells used for immunoblotting were cultured in T25 flasks
and when confluent, washed in PBS and then lysed in situ with 200 μl of
mammalian protein extraction reagent (M-Per, Pierce, Loughborough, UK)
containing complete protease inhibitor tablets (Roche) for 30 min at room
temperature. Protein levels in the lysates were assessed using a BCA assay

Cell Death and Disease

CO protects against amyloid peptide toxicity
NT Hettiarachchi et al
Intracellular K\(^+\) suppresses the system and hyperfilm ECL (Merck, UK).

Appropriate anti-rabbit or anti-mouse horse radish peroxidase-conjugated secondary antibodies were used for the detection of phospho-acetyl-CoA carboxylase (ACC), phospho-acetyl-CoA carboxylase (phospho-ACC) at room temperature. Bonferroni post-test, as appropriate.

Statistical analysis. Data are shown as mean ± S.E.M. Statistical analysis was carried out using one-way ANOVA followed by either the Dunnett’s or Bonferroni post-test, as appropriate. P values of less than 0.05 were considered significant. CellEvent results were analyzed using a two-way ANOVA followed by a Bonferroni post-test.

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