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REVIEW

Carbon monoxide and the CNS: challenges and achievements

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Haem oxygenase (HO) and its product carbon monoxide (CO) are associated with cytoprotection and maintenance of homeostasis in several different organs and tissues. This review focuses upon the role of exogenous and endogenous CO (via HO activity and expression) in various CNS pathologies, based upon data from experimental models, as well as from some clinical data on human patients. The pathophysiological conditions reviewed are cerebral ischaemia, chronic neurodegenerative diseases (Alzheimer's and Parkinson's diseases), multiple sclerosis and pain. Among these pathophysiological conditions, a variety of cellular mechanisms and processes are considered, namely cytoprotection, cell death, inflammation, cell metabolism, cellular redox responses and vasomodulation, as well as the different targeted neural cells. Finally, novel potential methods and strategies for delivering exogenous CO as a drug are discussed, particularly approaches based upon CO-releasing molecules, their limitations and challenges. The diagnostic and prognostic value of HO expression in clinical use for brain pathologies is also addressed.

LINKED ARTICLES
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Abbreviations
AD, Alzheimer's disease; BBB, blood–brain barrier; BK_{Ca}, large-conductance Ca^{2+}-activated K^+ channels; CMVEC, cerebral microvascular endothelial cell; CORM, carbon monoxide-releasing molecule; DN, dopaminergic neurone; EAE, experimental autoimmune encephalomyelitis; HO, haem oxygenase; IPC, ischaemic pre-conditioning; MCAO, middle cerebral artery occlusion; MPP, 1-methyl-4-phenylpyridinium; MS, multiple sclerosis; PD, Parkinson's disease; ROS, reactive oxygen species; sGC, soluble guanylyl cyclase

Historical aspects

Carbon monoxide (CO) is commonly considered to be toxic because of its high affinity for haem proteins, which can compromise oxygen delivery in tissues, via formation of carboxyhaemoglobin (Bernard, 1857; Haldane, 1895). Claude Bernard was the first to publish an accurate description of the physiology of CO poisoning (Bernard, 1857). About one century later, CO was also described as cytotoxic by limiting oxidative phosphorylation in cells, via the inhibition of cytochrome *c* oxidase (Wainio and Greenless, 1960; Savolainen *et al.*, 1980; see also Piantadosi, 2002).

Later, CO was recognized as an endogenous molecule in 1949, when Sjostrand (1949) identified this gas as a natural metabolite in the exhaled air of healthy humans. Nevertheless, it was only in 1968 that CO was identified as a product

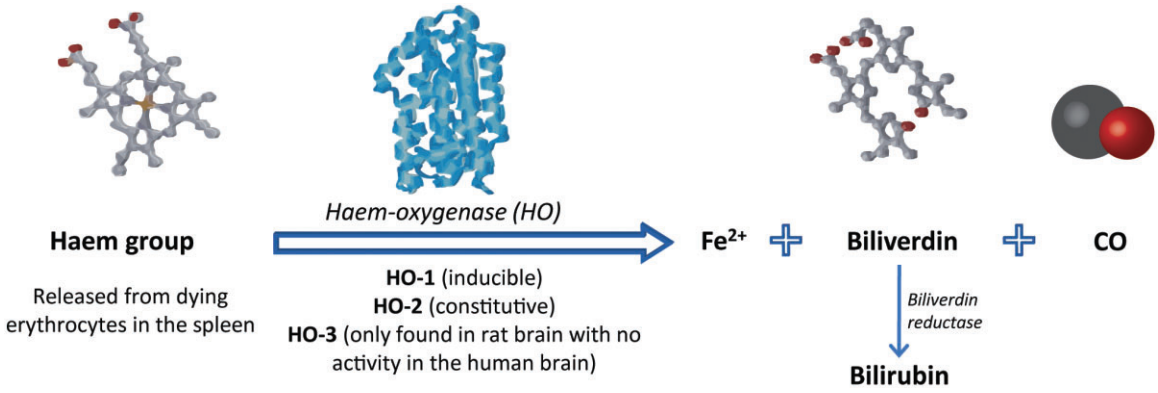

Figure 1

The enzyme haem oxygenase (HO) metabolizes the haem group, giving rise to free iron, biliverdin (rapidly converted to bilirubin) and carbon monoxide.

of haem catabolism by haem oxygenase (HO) (Tenhunen *et al.*, 1968; enzyme nomenclature follows Alexander *et al.*, 2013a), along with free iron and biliverdin (Figure 1).

In 1988, Harbin and colleagues assessed the neurophysiological effects of CO exposure, concluding that acute and low levels of CO exposure were not neurotoxic in normal healthy men (Harbin *et al.*, 1988). In 1993,, CO was accepted as a signalling molecule, being considered a neurotransmitter (Verma *et al.*, 1993). In the beginning of the new millennium, the first therapeutic actions of CO, as a vasomodulator (Sammut *et al.*, 1998), as an anti-inflammatory (Otterbein *et al.*, 2000) and as an anti-apoptotic factor (Brouard *et al.*, 2000) were described. Since then, several distinct applications of CO have been explored, namely in organ transplantation, as a cardioprotective, anti-inflammatory and anti-apoptotic molecule, and for limiting cell proliferation, in the particular case of atherosclerosis (see Motterlini and Otterbein, 2010). The first applied patent for the use of CO in medicine was in 2003 (Yale University: WO03094932A1; 2003; for further information on CO-based patents, see Zuckerbraun, 2008; Bannenberg and Vieira, 2009).

Haem oxygenase

HO can be found in two main isoforms: HO-1 or inducible and HO-2 or constitutive. Both respond to stress by increasing their expression or activity respectively (Ryter *et al.*, 2006). A further isoform, HO-3, has been found only in the rat brain (McCoubrey *et al.*, 1997) and not in the human. HO is expressed/activated in response to a wide range of different cellular stress conditions, such as oxidative stress, hyperthermia, hypothermia, ischaemia, hypoxia, hyperoxia, inflammation or exposure to UV light(Gozzelino *et al.*, 2010). HO plays a crucial role in the redox state of the cell and is crucial for cellular maintenance and survival in many organ systems, such as brain (Dore, 2002), heart (Piantadosi *et al.*, 2008), intestine (Nakao *et al.*, 2008), liver (Babu *et al.*, 2007) and lung (Morse *et al.*, 2009). The contribution of HO to the maintenance of both tissue homeostasis and cytoprotection is due to two main actions: (i) the removal of the haem group (originating from dying cells or from haemoglobin following haemorrhage) and (ii) the biological activity of HO products (Grochot-Przeczek *et al.*, 2012). It is worth noting that under stress, haem-containing proteins can release free haem, which becomes a potent oxidising agent through the Fenton reaction. For this reason, the catabolism of free haem by HO is crucial for maintaining tissue homeostasis and cytoprotection (see Gozzelino *et al.*, 2010).

HO and the CNS

In the brain, basal HO-1 expression is low, whereas under stress stimulation, it increases in neuronal, glial and endothelial cells. Likewise, constitutive expression of HO-2 is mainly distributed in mammalian neuraxis, but its expression can also increase following damaging stimuli (Schipper, 2004a,b), such as in hypoxic–ischaemic insult (Sutherland *et al.*, 2009). In several brain pathologies, HO expression and activity can be involved in the modulation of disease development, as well as in the re-establishment of tissue homeostasis.

HO levels as potential biomarkers in the CNS

The use of HO protein as a biomarker for brain damage presents some limitations. Firstly, increased levels of HO-1 in the human serum are not brain-specific and can indicate systemic inflammation and/or tissue damage. Secondly, although it is widely accepted that HO expression is associated with neuroprotection, glial cytoprotection and anti-inflammatory events, HO responds to several different stress stimuli. Likewise, increased expression of HO indicates pathological changes in the tissue. It is, therefore, quite difficult to interpret high-risk changes in the expression of HO and to decide if it is a biomarker of pathological processes or a predictor of a favourable outcome.

For example, the levels of HO-1 in the CSF of infants and children after severe traumatic brain injury are higher (Cousar *et al.*, 2006). Likewise, the level of HO-1 protein is a promising serum biomarker for early detection of Alzheimer's disease (AD), as they seem to increase in patients with AD and

mild cognitive impairment (Mueller *et al.*, 2010). An example showing that HO-1 levels can be used as a diagnostic and prognostic biomarker is during hypothermia treatment following haemorrhagic brain injury in a rat model (Yao *et al.*, 2011). The brain cooling-induced decrease on HO-1 expression was associated with an attenuation of oedema formation and a decrease of haem concentration (Yao *et al.*, 2011). Thus, in these three cases, HO-1 expression is associated with the development of pathology and can be used as a diagnostic biomarker. On the contrary, in experimental models of cardiovascular diseases and cerebral ischaemia, lower levels or deletion of HO-1 and -2 expressions are related to a worse outcome (see the following sections). Therefore, enhanced expression of HO could be associated with a favourable outcome being a prognostic biomarker. In summary, HO-1 and HO-2 levels display a potential and promising diagnostic and prognostic value as biomarkers in humans, although further studies are urgently necessary.

In the following sections, examples relating cerebral pathologies and HO are described in a systematic way.

Cerebrovascular diseases: ischaemia and reperfusion

Cerebral ischaemia is the main cause of brain damage and the third major cause of death in Western societies. In adults, it is mainly due to stroke, whereas in infants, it is caused by perinatal complications, particularly birth asphyxia. Cerebral damage is the result of oxygen and tissue energy depletion, leading to acidosis, exacerbated inflammation, glutamate excitotoxicity, oxidative stress and ultimately neural cell death (Dirnagl *et al.*, 2009).

Increasing data indicate that HO-1 activity is crucial for tissue protection and regeneration following cerebral ischaemia. In humans, there is a long-term increase in the expression of HO-1 following focal cerebral infarctions and traumatic brain injury (Beschorner *et al.*, 2000). On the contrary, in a rat model of transient cerebral ischaemia, reduction of HO-1 expression was associated with more severe neurodegeneration (Moreira *et al.*, 2007). Protection by ischaemic pre-conditioning (IPC) against permanent ischaemic brain injury is dependent on HO-1 expression, as IPC-promoted neuroprotection was abolished in HO-1 gene-deleted mice (Zeynalov and Dore, 2009; Zeynalov *et al.*, 2009). Likewise, overexpression of HO-1 by adenovirus vector treatment attenuated brain damage after focal cerebral ischaemia in rats (Chao *et al.*, 2013).

Modulation of cerebrovasodilation by HO

In neonates, recurrent seizures may result from meningitis, haemorrhage, asphyxia, and hypoxia or metabolic disorders. Neonatal seizures may promote neuronal damage and susceptibility to epilepsy in survivors. Both HO-1 and HO-2 activities in astrocytes, neurons, endothelial cells and smooth muscle cells (in cerebral vessels) are involved in the modulation of cerebral blood flow and vasodilation during seizures (Parfenova *et al.*, 2003; 2012a; Basuroy *et al.*, 2006; Xi *et al.*, 2011). Moreover, ionotropic glutamate receptors mediate HO activation and endogenous production of CO, which increases cerebral blood flow, essential for maintaining brain homeostasis and neuronal survival during seizures (Parfenova *et al.*, 2012b).

HO and AD

Despite the increasing amount of data demonstrating HO as a widespread cytoprotective enzyme, its homeostatic and neuroprotective role in AD is somewhat controversial. AD is associated with an increased deposition of redox-active iron, chronic oxidative stress and mitochondrial malfunctioning, which are implicated in the development of this pathological disorder. Indeed, in experimental models, glial overexpression of HO-1 promoted mitochondrial oxidative stress (Song *et al.*, 2006) and mediated mitochondrial membrane damage and autophagy in astrocytes (Zukor *et al.*, 2009). Additionally, in mouse brain, long-term overexpression of HO-1 induced toxic tau accumulation (Hui *et al.*, 2011) and increased deposits of glial iron (Song *et al.*, 2012).

On the contrary, HO expression appears to be involved in reduction of brain oxidative stress. In an ageing canine model, which develops cognitive dysfunction and neuropathology similar to those in human AD patients, atorvastatin-induced up-regulation of HO was associated with reduced oxidative stress (Butterfield *et al.*, 2012). In the same canine model, brain oxidative stress biomarkers (protein carbonyl, 3-nitrotyrosine and levels of products of lipid peroxidation) were attenuated following enriched environment-antioxidant-fortified feeding, which was strongly associated with an enhancement of HO-1 protein levels (Opii *et al.*, 2008).

HO suppressor factors, such as α1-antitrypsin, may also play a role in the development of AD, as Maes *et al.* (2006) have found significantly augmented plasma HO suppressor activity in AD patients, relative to healthy elderly subjects.

As previously mentioned, HO levels were increased in the serum of AD patients, and could be being a potential diagnostic biomarker (Mueller *et al.*, 2010). In addition, HO post-translational modification might also be involved in the development of AD. Barone and colleagues found that HO-1 protein levels were significantly increased in the hippocampus of AD subjects, whereas HO-2 protein levels were significantly decreased in both AD and mild cognitive impairment hippocampi. Serine phosphorylation and increased oxidative, post-translational, modifications of HO-1 were also found in the hippocampus of AD patients (Barone *et al.*, 2012). Controversially, it was also observed that HO-1 protein levels are lower in *post mortem* specimens of CSF (see Schipper 2000).

Thus, HO isoforms and protein post-translational modifications might also play a role in the debate between neuroprotective versus neurotoxic effects of HO activity in AD.

HO and Parkinson's disease (PD)

Oxidative stress, accumulation of Lewy bodies and decrease of mitochondrial complex I activity are common features occurring in nigral dopaminergic neurons (DNs) during pathological development of PD. In *post mortem* human brain specimens collected from PD patients, HO-1 expression was assessed by immunohistochemistry. In the substantia nigra of both PD and control specimens, moderate HO-1 immunoreactivity was consistently observed in DNs, while the fraction of GFAP-positive astroglia expressing HO-1 in PD substantia nigra was significantly greater in PD patients (Schipper *et al.*, 1998). Likewise, expression of HO-1 measured by microarray analysis was enhanced following oxidative stress in DNs (Yoo

et al., 2003). Despite the association of HO-1 expression with PD development, HO-1 activity emerges as involved with neuroprotection. For instance, in a rat model of MPP^+ (1-methyl-4-phenylpyridinium)-induced PD, local injection of adenovirus containing human *HO-1* gene increased the survival rate of DNs and reduced the production of TNF-α (Hung *et al.*, 2008). Using an *in vitro* model of rat midbrain slice culture, in which DNs were induced to die by IFN-γ/LPS treatment, surviving neurons displayed more robust expression of HO-1, whereas treatment with a HO-1 inhibitor, zinc protoporphyrin IX, increased cell death (Kurauchi *et al.*, 2009). Fibroblast growth factor 9 prevented MPP-induced nigral dopaminergic neuronal death via up-regulation of HO-1 (Huang and Chuang, 2010). In the autosomal recessive form of PD, due to the PINK1 G309D mutation, there is an impairment of HO-1 production in response to oxidative stress (Chien *et al.*, 2011). In addition, HO-1 activity also seems to be associated with modulation of proteasome degradation, whose activity is decreased in patients with PD. Indeed, misfolding proteins promote neuronal toxic stimuli, which induce HO-1 expression, and, in turn, prevent intracellular accumulation of protein aggregates and inclusions in human neuroblastoma M17 cells (Song *et al.*, 2009). Controversially, HO-1 knockout mice treated with MPP i.p. injection for inducing PD presented the same levels of dopaminergic degeneration and severity of gliosis as control animals (Innamorato *et al.*, 2010).

In summary, although HO activity is associated with cytoprotection and neuroprotection, some authors have suggested that it is implicated in neurotoxicity and should be a therapeutic target for chronic neurodegenerative diseases (AD and PD), namely through the prevention of its expression and/or activity for avoiding iron accumulation. Indeed, Schipper *et al.* (2009) suggested the suppression of glial HO-1 activity as a potential therapeutic strategy for treating AD. Furthermore, the levels of ferritin protein are crucial for maintaining a functional cellular iron storage, whose role must be coupled with HO activity. Ferritin is a very important protein with a dual role of protecting the cell against the oxidative stress caused by free iron, yet allowing access to it. There are two isoforms, L and H, distributed throughout the tissues. L-ferritin has iron nucleation properties and a mutation on this chain leads to iron deposition in cerebellum, basal ganglia and motor cortex, causing an autosomal dominant inherited disorder (neuroferritinopathy) (Lehn *et al.*, 2012). Additionally, H-ferritin mutations lead to a propensity to oxidative stress, notwithstanding normal iron concentration, as the L-ferritin compensates for the loss of H-ferritin. Thus, one can also speculate that, depending upon the ferritin levels and activity, HO could promote cytoprotection or exacerbation of damage. Indeed, Thompson *et al.* (2003) generated a mouse model for AD and PD, based upon a deficiency on H-ferritin, reinforcing the deleterious role of iron in neurodegenerative diseases. Another important discovery is the existence of mitochondrial ferritin, which is expressed only in the testis and brain (Yang *et al.*, 2013). Despite the lack of data until this date, mitochondrial ferritin is considered to be associated with neuroprotection against neurodegeneration in PD and AD. Thus, the effects of HO on neurodegenerative diseases need to be studied conjointly with ferritin activity.

Neuroinflammation and multiple sclerosis (MS)

During the past decade, several reports have demonstrated that HO activity can also modulate neuroinflammation. HO-1 appears to be involved in the modulation of neuroinflammation because whenever its transcription factor Nrf-2 is knocked out, mice are hypersensitive to LPS-induced neuroinflammation (Innamorato *et al.*, 2008). Still, molecules exerting anti-neuroinflammatory effects, such as dimethyl fumarate (Lin *et al.*, 2011), cyclopentenone prostaglandins (Zhuang *et al.*, 2003b) and 6,4′-dihydroxy-7-methoxyflavanone (Li *et al.*, 2012) act by increasing expression of HO-1.

MS is an autoimmune disease affecting the CNS with inflammatory lesions, demyelination and axonal loss (Fagone *et al.*, 2012). In 2001, the protective and anti-inflammatory role of HO-1 activity in an experimental model of MS, experimental autoimmune encephalomyelitis (EAE) was first shown. Pharmacological induction of HO-1 with haemin effectively inhibited EAE, whereas prevention of HO-1 activity with tin mesoporphyrin exacerbated EAE (Liu *et al.*, 2001). Later, the same effect was demonstrated by genetic inhibition of HO-1, EAE induction in HO-1 knockout mice enhanced CNS demyelination, paralysis and mortality (Chora *et al.*, 2007). Likewise, MS patients present reduced levels of HO-1 expression in peripheral blood mononuclear cells, and during the exacerbation of the disease, there is a significant down regulation of this enzyme (Fagone *et al.*, 2013). In contrast, there is also evidence that overexpression of HO-1 in glial cells was toxic by promoting mitochondrial oxidative stress and damage due to free iron accumulation (Mehindate *et al.*, 2001), and this effect could be reversed by the addition of the iron chelator deferoxamine (Song *et al.*, 2006). Likewise, in astrocytes of spinal cord from MS, patient there were higher levels of HO-1 than in astrocytes from control subjects (Mehindate *et al.*, 2001).

Pain

Carvalho and colleagues proposed the HO-CO-cGMP pathway to be involved in the nociception caused by an acute painful stimulus without inflammation. The administration of pharmacological inhibitor or substrate of HO and soluble guanylyl cyclase (sGC) inhibitor have shown that the antinociceptive action is reduced whenever HO activity is prevented, this effect being dependent upon sGC (Carvalho *et al.*, 2011).

HO in neuroprotection induced by naturally occurring compounds

Epidemiological studies have revealed a reduced incidence of cardiovascular and neurodegeneration risk associated with consumers of specific foods, such berry fruits and red wine. Furthermore, a wide variety of natural compounds extracted from plants or fruits are claimed to promote neuroprotection through modulation of HO-1 expression and/or activity. In 2002, it was first described that in astrocytes, curcumin induces HO-1 expression and activity in a glutathione-independent way (Scapagnini *et al.*, 2002). Since then, several publications have shown, in cultures of neurons and astrocytes, that curcumin protects against inflammation, oxidative

Table 1

Natural compounds extracted from plants or fruits that promote neuroprotection through modulation of HO-1 expression and/or activity

Compound	Model	Observations	Reference
Ginkgo biloba	Middle cerebral artery occlusion (transient) and permanent ischaemic stroke	HO-1 KO mice lost beneficial effects	Saleem *et al.*, 2008; Shah *et al.*, 2011
Ginkgo biloba	Primary culture of neurons are challenged with oxidative stress and excitotoxicity	HO-1 deleted derived neurons are not protected against cell death	Nada and Shah, 2012
Resveratrol	Ischaemia–reperfusion in rats	Resveratrol attenuated brain tissue damage and increased HO-1 expression	Ren *et al.*, 2011
Flavanol(-)-epicatechin	Middle cerebral artery occlusion and neuronal culture	Deletion of HO-1 abolished neuroprotective role of this flavanol	Shah *et al.*, 2011
Sevoflurane	Rat model of focal cerebral ischaemia	Induction of HO-1 up-regulation during post-conditioning	Ye *et al.*, 2012
Triterpenoid	Global ischaemia in rat Focal ischaemia in mice Oxygen-glucose deprivation in neuronal cultures	8 times increase of HO-1 expression in neuronal culture, *in vivo* enhanced HO-1 expression and reduced neurological dysfunction and infarct volume	Zhang *et al.*, 2012
Octreotide	Middle cerebral artery occlusion	Increased expression of HO-1	Chen *et al.*, 2012

damage and cell death (Table 1). Ginkgo biloba, which is an extract used in traditional Chinese medicine, has been widely described as a neuroprotective substance. In Table 1, there are several examples showing the involvement of HO in Ginkgo biloba-induced neuroprotection using *in vitro* and *in vivo* models. Resveratrol, which is a component of red wine associated with cardioprotection and neuroprotection, was demonstrated to confer its healthy properties by HO-1 activation *in vitro* and *in vivo* (Zhuang *et al.*, 2003a; Sakata *et al.*, 2010), (Ren *et al.*, 2011). Finally, other natural occurring compounds, such as flavanol(–)-epicatechin, sevoflurane, triterpenoid and octreotide, are also implicated in neuroprotection via HO-1 activation (Table 1).

Carbon monoxide and carbon monoxide-releasing molecules (CORMs)

During the past two decades, many biological functions of CO have been described and great efforts are being made to develop its use for human health. The potential clinical application of inhaled carbon monoxide presents several disadvantages: (i) inhaled CO is not tissue specific; (ii) CO gas is, at least partly, delivered in the body through blood plasma flow and carboxyhaemoglobin, leads to partial systemic hypoxia and toxicity; and (iii) the need of hospital facilities with technical devices for CO inhalation and monitoring oxygen blood levels. To overcome these limitations, great efforts have been taken by chemists to create pro-drugs by synthesizing molecules able to deliver CO, which were first called carbon monoxide-releasing molecules – CORMs (Motterlini *et al.*, 2002). Although a large number of CORMs were developed in the last decade, only few of them have shown proven and efficient beneficial biological effects in *in vivo* and *in vitro* systems. Several issues must be overcome in the development of CORMs, namely water insolubility, toxic chemical structures, promotion of high levels of carboxyhaemoglobin and chemical instability (for further review on their development, see Romao *et al.*, 2012). In the particular case of the CNS, the most studied pro-drugs were CORM-A1, CORM-2 and CORM-3. CORM-A1 ($[H_3BCO_2]Na_2$) is a boranocarbonate molecule (Motterlini *et al.*, 2005), whereas the transition metal-based molecules are CORM-2 $[Ru(CO)_3Cl_2]_2$, which is a dimer and insoluble in water (Motterlini *et al.*, 2002), and the water-soluble CORM-3 ($[Ru(CO)_3Cl(H_2NCH_2CO_2)]$) (Clark *et al.*, 2003). Furthermore, in the specific case of experimental cerebral malaria, a new ruthenium-based molecule was tested, ALF 492, presenting CORM-3 structure with methylthiogalactopyranoside ligand (Pena *et al.*, 2012). The molybdenum-based water-soluble molecule ALF 186 was shown to confer neuroprotection (Schallner *et al.*, 2013).

Developing drugs for brain pathologies is highly challenging due to its extreme importance and complexity, as well as due to the presence of blood–brain barrier (BBB), a biological barrier constituted by the endothelial cells of the blood capillaries together with associated astrocytic end-feet processes and perivascular neurons. The BBB serves to isolate the brain and decreases the risk of infection and the entrance of toxins. Although much work has been done on CORMs and the brain, the ability of any CORM to cross the BBB has not been fully clarified, while it is accepted that the released CO gas could cross biological membranes.

CO and the CNS

Exogenous administration of low levels of CO (as CO gas or as CORMs) has been explored as potential therapeutic factor in many different models of brain pathologies (Figure 2), which are described in this section.

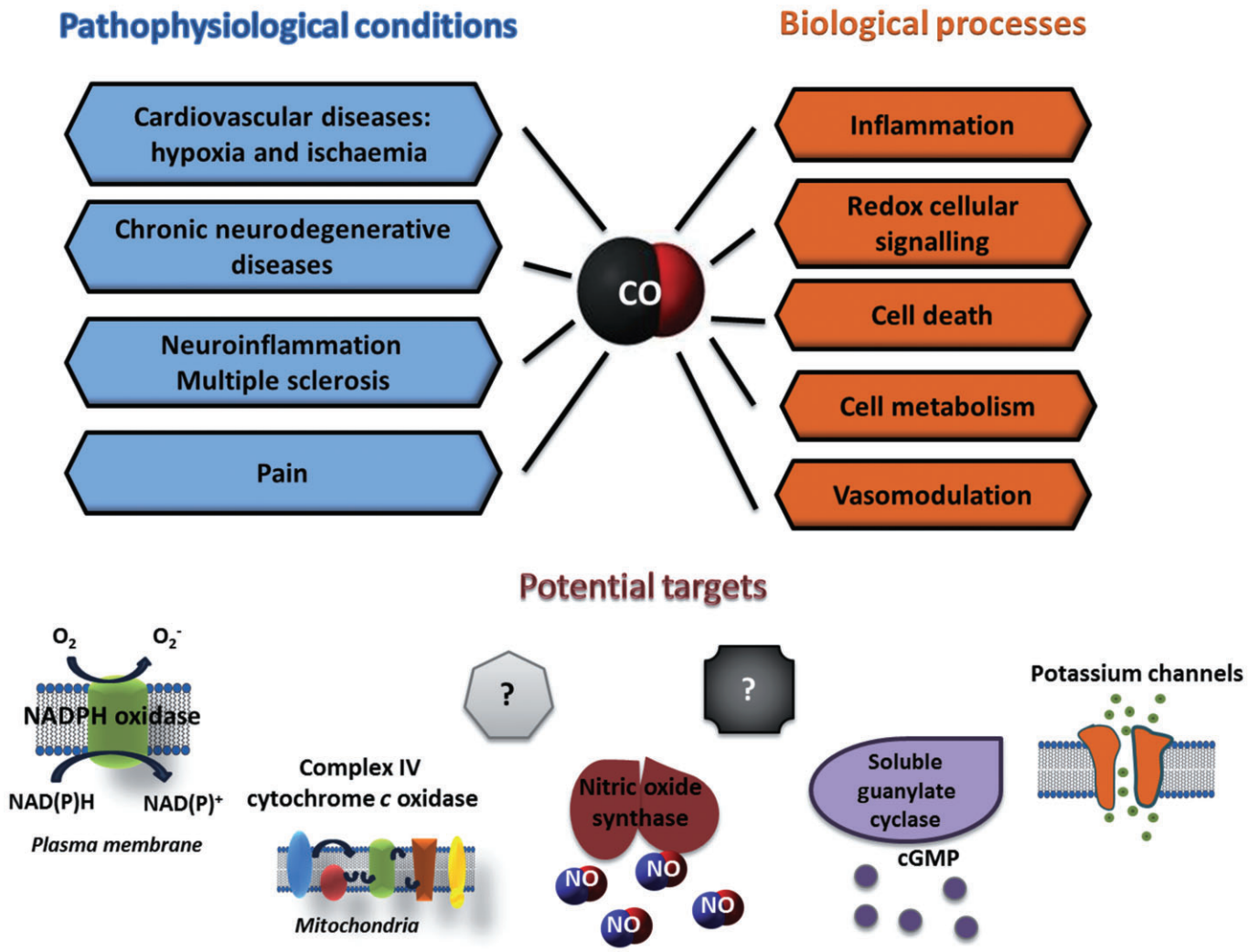

Figure 2

Schematic representation of the pathophysiological conditions, biological processes and potential targets for CO.

Cerebrovascular disease

Low levels of inhaled CO were beneficial against cerebral hypoxic and ischaemic insult in experimental rodent models. In mice, CO exposure at 250 ppm for 18 h immediately after permanent middle cerebral artery occlusion (MCAO) decreased the infarct volume by about 30% after 7 days (Wang *et al.*, 2011). Likewise, in a transient MCAO model (90 min of focal ischaemia followed by 48 h of reperfusion), inhalation of 125 ppm of CO immediately at the onset of reperfusion also decreased brain damage by about 30% after 48 h. Inhalation of CO at 250 ppm in the same model and conditions decreased brain damage by around 60%. Interestingly, when the CO inhalation is performed 1 h or 3 h after the reperfusion, there is still reduction in brain damage, by 70 and 30%, respectively (Zeynalov and Dore, 2009). In a rat model of haemorrhagic stroke, when CORM-3 was administered 5 min before or 3 days after, the intracerebral haemorrhage stimulus (injection of collagenase), inflammatory responses were decreased. The opposite effect was achieved when CORM-3 is injected 3 h after the haemorrhagic insult (Yabluchanskiy *et al.*, 2012). Thus, the time window for CO administration is a crucial aspect for its biological functions. In a perinatal rat model of cerebral hypoxia–ischaemia, CO exposure at 250 ppm for 1 h·day^{-1} on the 3 days prior to the ischaemic insult decreased apoptotic cell death in the hippocampus by 64% (Queiroga *et al.*, 2012b). In another perinatal experimental system, a piglet model of deep hypothermic circulatory arrest, mimicking the open-heart surgery procedures, inhalation of 280 ppm CO for 3 h, 1 day prior to surgery, limited cell death in the neocortex and hippocampus (Mahan *et al.*, 2012). In the *in vivo* retinal ganglion cell model of ischaemia and reperfusion, CO gas pre-conditioning (Biermann *et al.*, 2010) and post-conditioning (Schallner *et al.*, 2012) also promoted neuroprotection. Finally, CORM-A1 (2 mg·kg^{-1}, i.p.) administration 30 min before chemically induced seizures, protected against seizure-induced neonatal vascular injury in newborn piglets (Zimmermann *et al.*, 2007; Parfenova *et al.*, 2012a).

Multiple sclerosis

In the established model of MS, EAE in SJL mice, a prolonged prophylactic treatment with CORM-A1 reduced the incidence of the disease and attenuated the inflammatory infiltrations of the spinal cords (Fagone *et al.*, 2011). Exogenous CO administration (250 ppm) suppressed myelin-reactive immune cell activation within the CNS, contributing to the reduction of autoimmune neuroinflammation impairment (Chora *et al.*, 2007).

Pain

Pain is another aspect where CO has the potential of improving patient quality of life. Hervera and colleagues demonstrated that treating mice with CORM-2 and CORM-3 for 10–20 days following sciatic nerve injury improved the local antinociceptive effects of morphine and significantly reduced the main neuropathic pain symptoms, in a time-dependent manner. Furthermore, this CO effect is due to the reduction of spinal microglial activation and NOS1/NOS2 overexpression (Hervera *et al.*, 2012; 2013a,b).

Brain cells and carbon monoxide: in vitro *approaches*

Cellular consequences of ischaemia, such as excitotoxicity and oxidative stress, induce cell death and can be mimicked *in vitro*. In primary cultures of cerebellar granular neurons, CO gas limited neuronal cell death via ROS signalling and acting on NOS, sGC and the ATP-dependent mitochondrial K channel (Vieira *et al.*, 2008). Similarly, CO-induced neuroprotection was shown to be dependent upon sGC activity and cGMP production in the SH-SY5Y neuronal cell line and in retinal ganglion cells by using a novel CORM: ALF 186 (Schallner *et al.*, 2013).

Neuroprotection does not concern only neurons. Indeed, one must also target glial cells to achieve complete neuroprotection. The physiological role of astrocyte, microglia, oligodendrocytes and endothelial cells is the maintenance of brain homeostasis, metabolism and neuronal function. Therefore, modulation of glial cell function is crucial for promoting neuroprotection. Likewise, regulation of astrocytic metabolism and prevention of astrocytic apoptosis against oxidative stress is decisive for the maintenance of brain homeostasis. Indeed, CO gas limited astrocytic apoptosis by two distinct ways: (i) direct prevention of mitochondrial membrane permeabilization and the consequent release into the cytosol of pro-apoptotic factors (Queiroga *et al.*, 2010) and (ii) improvement of cellular metabolism and increase of oxidative phosphorylation and mitochondrial population (Almeida *et al.*, 2012).

Furthermore, excessive inflammatory responses can be detrimental and the modulation of inflammation in microglia by CO is very important for the control of neuroinflammation. Many studies of the CO anti-inflammatory effect have been carried out *in vitro* using BV-2 microglial cells. CORM-3 was shown by Bani-Hani and colleagues to decrease NO production and TNF-α release in response to LPS, thrombin and IFN-γ stimuli. inhibition of MAPKs, and PI3K, exacerbated the anti-inflammatory effect of CORM-3. On the opposite, sGC, NOS and HO activity had no influence on the mode of action of CORM-3 (Bani-Hani *et al.*, 2006a,b). Taking all together, the ability of CO to limit inflammatory response promotes neuronal survival and is important for CO-induced neuroprotection.

Inflammatory brain disease, oxidative stress or excitotoxicity (with excessive glutamate release) might damage cerebral vascular endothelial cells, leading to blood flow dysregulation and permeabilization of the BBB. Parfenova *et al* demonstrated that cerebral microvascular endothelial cells (CMVECs) contain HO-1 and HO-2 isoforms and their endogenous CO regulates vascular tone in response to glutamate (Parfenova *et al.*, 2001; 2003; Leffler *et al.*, 2011). Likewise, endogenous and exogenous CO prevents endothelial cell death via modulation of Nox4 NADPH activity (Basuroy *et al.*, 2009; 2011) (see the following section). Finally, CORM-A1 prevents BBB dysfunction by limiting glutamate-induced apoptosis and oxidative stress in CMEC (Basuroy *et al.*, 2013). It is possible that the astrocytic end-feet processes and perivascular neurons associated with BBB are the prime targets of CO's effects in the brain.

Pathways involved in CO signalling

Several pathways have been proposed to contribute to the cellular and biochemical mechanisms associated with the biological roles of CO (Figure 2). However, those biochemical pathways and the actual physiological target(s) of CO are still under vigorous discussion (Motterlini and Otterbein, 2010). CO is a rather chemically inert molecule, and in biological systems, it can only bind to transition metals present in several proteins (Boczkowski *et al.*, 2006), thus modulating their activity. In mammals, iron-containing haem-proteins are the most studied and documented targets for CO. Notably, CO can only bind to reduced Fe^{2+}, limiting the potential target proteins, in contrast to NO that binds to both Fe^{2+} and Fe^{3+} (Boczkowski *et al.*, 2006).

In the CNS, the pathways and potential targets of CO are still poorly understood with few available data published concerning the mechanisms by which CO confers neuroprotection, anti-neuroinflammation or vasomodulation and this lack is crucial to the development of more scientific research on this subject. This section focuses upon and discusses the existing data about the pathways used by CO in the brain.

sGC and NOS

One of the most studied pathways for CO is the activation of sGC and NOS. Nevertheless, the binding affinity of CO for sGC is still controversial under physiological conditions, as high concentrations of CO are usually required for activating sGC, compared with the much lower levels of NO that are needed for activating sGC. Regarding neuronal cells, activation of sGC and NOS and the respective production of cGMP and NO were shown to be important for CO-induced neuroprotection against excitotoxicity and ischaemic insult (Vieira *et al.*, 2008; Schallner *et al.*, 2013). In a model of permanent ischaemic stroke, the protective role of HO-1 is correlated with higher levels of endothelial NOS expression in the brain (Shah *et al.*, 2011). Likewise, in a neuroinflammatory model, CO regulates inflammation in microgial cells by modulating NO production (Bani-Hani *et al.*, 2006a,b). Still, increased levels of cGMP appeared to be downstream to endogenous

CO production in astrocytes (Imuta *et al.*, 2007), whereas in cerebral microvessels, cGMP signalling appeared to be upstream of CO modulation, because glutamate-induced NOS activation led to CO production via cGMP signalling (Leffler *et al.*, 2005).

Finally, CO appears also to modulate pain through NO signalling. The antinociceptive effects of morphine and agonists of μ-opioid receptors, δ-opioid receptors and cannabinoid CB_2 receptors are enhanced by CO (CORM-2 and CORM-3) in a NO-dependent fashion, during chronic inflammatory and neuropathic pain (Hervera *et al.*, 2013a,b).

Reactive oxygen species (ROS) signalling

It is increasingly accepted in several cell and tissue models that the mediation of CO-induced cytoprotection is via ROS generation and signalling (see Bilban *et al.*, 2008; Queiroga *et al.*, 2012a). At least two cellular proteins are recognized as being directly implicated in cell redox signalling by CO: cytochrome *c* oxidase (mitochondrial respiratory complex IV) and NAD(P)H oxidase (plasmatic membrane).

Cytochrome *c* oxidase is the main described target for the cytotoxic effects of CO as, by binding to cytochrome *c* oxidase, CO blocks mitochondrial respiration, promoting cell death (Wainio and Greenless, 1960; Savolainen *et al.*, 1980; Alonso *et al.*, 2003). Furthermore, endogenous CO can also control and inhibit cellular respiration through acting on cytochrome *c* oxidase (D'Amico *et al.*, 2006). In neural cells, namely astrocytes, low concentrations of CO present a two-step response regarding cytochrome *c* oxidase activity. During the first minutes following CO treatment, there is a slight decrease in the cytochrome *c* oxidase activity, while after 30 min (and up to 24 h), specific activity of cytochrome *c* oxidase increases (Almeida *et al.*, 2012). Thus, these data indicate a direct action of CO on complex IV of mitochondrial respiratory chain and reinforces the hypotheses claiming that ROS production occurs at complex III level, due to electron accumulation whenever complex IV is inhibited. Likewise, in non-synaptic isolated mitochondria from rat brain cortex, CO promoted ROS generation (Queiroga *et al.*, 2010), and the use of β-carotene for limiting ROS levels prevented the anti-apoptotic effect of CO in astrocytes, as well as the CO-induced protection against mitochondrial membrane permeabilization (Queiroga *et al.*, 2010). In primary cultures of cerebellar granular neurons, small amounts of ROS are produced upon CO treatment and, when ROS generation was prevented by butyl-hydroxytoluene, the neuroprotective effect of CO was reversed, indicating the essential role of ROS as signalling factors (Vieira *et al.*, 2008).

In inflammatory brain diseases, NADPH oxidase, particularly its major isoform Nox4, generates ROS, which can initiate both death and survival pathways in TNF-α-challenged CMVECs. Endogenous and exogenous CO limits the production of superoxide anion by Nox4 NADPH, preventing endothelial cell death caused by TNF-α-induced oxidative stress (Basuroy *et al.*, 2009). Nox4 NADPH-derived ROS also initiated a cell survival mechanism, by increasing production of CO by constitutive HO-2 (Basuroy *et al.*, 2011). The ROS-dependent cell survival pathway is mediated by TNF-α, Akt, ERK1/2 and p38 MAPK signalling pathways (Basuroy *et al.*, 2011). Therefore, there might be a feedback control of ROS production regulated by CO, whereby NADPH oxidase produced ROS that increased CO generation, which, in turn, prevented NADPH oxidase activity, its excessive production of superoxide anion and oxidative stress.

Outside the nervous system, there are other potential pathways for biological CO action, related to ROS signalling and mitochondria. In cardiomyocytes, CO-induced mitochondrial ROS production may control mitochondrial biogenesis, leading to cytoprotection (Suliman *et al.*, 2007a,b). Likewise, in isolated heart mitochondria, CORM-3 limits excessive mitochondrial ROS production and avoids oxidative stress by inducing a mild-uncoupling state, while complex II seems to be the target of CO as inhibition of complex II (malonate addition) reversed the CO-induced augmentation of oxygen consumption and the uncoupling effect (Lo Iacono *et al.*, 2011). In contrast, in a liver system, CO has been described as a cytoprotective molecule by targeting cytochrome P450 and limiting excessive ROS production and oxidative stress-induced cell death. The best-described example is the isoform cytochrome P450 2E1, which is involved in acetaminophen (paracetamol) hepatotoxicity (Gong *et al.*, 2004). Based upon the data derived from other organs and tissues, neuroscientists should explore other potential targets and pathways for the well-accepted beneficial effects of CO in the brain.

CO and potassium channels

In 2003, it was shown by Tang and colleagues that large-conductance calcium-dependent potassium channels (BK_{Ca}; $K_{Ca}1.1$; channel nomenclature follows Alexander *et al.*, 2013b) contain a conserved haem-binding sequence motif, which can bind covalently to haem, regulating its channel activity (Tang *et al.*, 2003). One year later, HO-2-derived CO was demonstrated to modulate the BK_{Ca} channels, which are important for sensing oxygen levels (Williams *et al.*, 2004; Jaggar *et al.*, 2005). Furthermore, endogenous CO may modulate cerebral microvasculature by activating these channels (Jaggar *et al.*, 2005). Particularly, astrocytic HO-2-derived CO causes glutamatergic dilation of pial arterioles, by activating smooth muscle cell BK_{Ca} channels (Leffler *et al.*, 2011). Thus, one can speculate that CO binds directly to BK_{Ca} channel-bound haem to control dilation and constriction of vasculature (Leffler *et al.*, 2011).

Future challenges for CO administration

The first challenge to the clinical use of CO in cerebral pathologies is the lack of information about its mode of action. Although many different mechanisms for the cellular and biochemical pathways of CO action have been described, the precise underlying signalling mechanisms and the exact molecular target(s) of CO are poorly defined. It is worth noting that elucidating the potential protein targets of CO under physiological conditions is extremely complex as CO might bind to its target on a dynamic and transitory way. Furthermore, CO directly competes with oxygen for binding to proteins; thus, tissue and cellular oxygen levels also influence the system used to study CO targets under physiological conditions. Based on the fact that CO seems to mimic pre-

conditioning, promoting a tissue tolerance state, one might explore the classical activator and transducer factors involved in pre-conditioning and CO. For instance, pre-conditioning stimulus leads to up-regulation of VEGF (Wick *et al.*, 2002; Laudenbach *et al.*, 2007), activation of hypoxia inducible factor (HIF-1) (Ratan *et al.*, 2004; Chu *et al.*, 2010) or expression of erythropoietin (Ruscher *et al.*, 2002), these factors are promising candidates for CO-related pathways. Indeed, in macrophages, CO has been described as stabilizing HIF-1 (Chin *et al.*, 2007).

The second challenge concerning CO administration is achieving the best way to specifically deliver CO to the target tissue, avoiding high concentrations of carboxyhaemoglobin. Many studies have been made to develop CORMs to avoid the systemic toxicity related to carboxyhaemoglobin (see Romao *et al.*, 2012; Zobi, 2013) and there are now CORMs which induce different levels of carboxyhaemoglobin, Whereas exposure to CO gas and CORM-A1 administration induced similar levels of carboxyhaemoglobin (Otterbein *et al.*, 1999; Ryan *et al.*, 2006), very low levels of carboxyhaemoglobin were observed with CORM-3 (Guo *et al.*, 2004). Nevertheless, how and where to give CORMs to deliver CO efficiently is still a matter of intensive research. Likewise, chemical modifications of CORMs are under progress to target these molecules to a specific organ or cell type (Fagone *et al.*, 2012). So far, the best example is ALF 794 which specifically targets the liver against acute injury (Marques *et al.*, 2012). Still, several questions remain unanswered: How is the CORM transported in blood? Does the CORM bind to any protein present in the blood in order to maintain its stability? Does the CORM need to cross the cellular plasma membrane? Is CO actually delivered in the extracellular space, arriving intracellularly by membrane diffusion? It is clear that many further studies are needed before we can know how the existing CORMs act under physiological conditions. Still, the development of new molecules with optimal control of CO delivery (locus and kinetics) is also crucial for the progress of CO as a novel therapeutic agent for medical applications.

Furthermore, in the brain, another vital biological challenge exists in the shape of the BBB. Several brain studies have been performed *in vivo* using CORM-3 and CORM-A1 with promising results. Although it is not precisely confirmed that these CORMs are able to cross the BBB, CO does enter the brain and acts as a cytoprotective molecule (Zimmermann *et al.*, 2007; Parfenova *et al.*, 2012a; Yabluchanskiy *et al.*, 2012).

The time window for CO administration is essential to optimise the outcome and this factor depends upon the pathophysiological situation. Pre-conditioning is one of the processes induced by CO, where CO stimulates endogenous cellular pathways of protection (anti-inflammatory, anti-apoptotic, pro-survival, pro-homeostatic etc.) (Bilban *et al.*, 2008; Piantadosi *et al.*, 2008; Queiroga *et al.*, 2012b). In this case, the therapeutic strategy consists of CO administration prior to the injury; for instance, in patients at high risk of developing cerebral ischaemia (before major cardiac surgery, high-risk newborn infants, ageing patients with cardiovascular complications and risk of ischaemic stroke). Moreover, during the development of chronic diseases, such as AD, PD or MS, exogenous CO can be used as a pre-conditioning agent (Fagone *et al.*, 2011). Other evidence shows that CO has beneficial effects in acute injury, suggesting that CO could be applied after injury, as a post-conditioning strategy, as described in cerebral ischaemia, intracerebral haemorrhage and seizures (Zeynalov and Dore, 2009; Wang *et al.*, 2011; Yabluchanskiy *et al.*, 2012).

Final conclusions

There is good evidence supporting the protective role of CO (and the enzyme catalysing its biosynthesis, HO) in the CNS in the context of several pathologies, including cerebrovascular diseases, neuroinflammation, MS, pain, AD and PD.

Essential to the therapeutic use of CO is further development of sources of CO, other than CO gas, to overcome the problem of carboxyhaemoglobin toxicity. CORMs have been increasingly used with successful and interesting results. Nevertheless, it was inhaled CO that was first proved to be safe and tolerable in humans.

Independent of the route of administration and regardless of the cell type, CO appears to modulate several important cellular enzyme systems, including cytochrome *c* oxidase, NOS, sGC and NADPH oxidase. Other targets, such as ROS signalling and mitochondria, are also significant components of the actions of CO. Nevertheless, further research is urgently needed to define more precisely the biological target(s) and pathways of this gasotransmitter. In conclusion, CO has travelled far, from being an invisible enemy to becoming a possible therapeutic solution.

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Conflict of interest

There is no conflict of interest.

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