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Carbon Monoxide and Central Nervous System: Challenges and Achievements

Abbreviated title: CO and central nervous system

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Summary

Haem oxygenase (HO) and its product carbon monoxide (CO) are associated with cytoprotection and maintenance of homeostasis in several distinct organs and tissues. This review focuses on the role of exogenous and endogenous CO (*via* HO activity and expression) in various central nervous system pathologies, based on data from experimental models, as well as from some clinical data on human patients. The described and revised pathophysiological conditions are cerebral ischemia, chronic neurodegenerative diseases (Alzheimer and Parkinson diseases), multiple sclerosis and pain. Among these pathophysiological conditions, some 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, in particular approaches based on CO-releasing molecules, their limitations and challenges. The diagnostic and prognostic value of haem oxygenase expression in clinical use for brain pathologies is also addressed.

Keywords: Carbon Monoxide; CO Releasing Molecules; neuroprotection, neuroinflammation, brain, astrocytes, neurons, microglia, neurodegeneration, ischemia

Abbreviation List: AD - Alzheimer disease; BBB – blood-brain barrier; BK(Ca) – large conductance Ca^{2+}-activated K^{+}; CMVEC – cerebral microvascular endothelial cells; CNS – central nervous system; CO – carbon monoxide; CORM – carbon monoxide releasing molecules; DHCA – deep hypothermic circulatory arrest; EAE – experimental autoimmune encephalomyelitis; FBF-9 – fibroblast growth factor 9; INF-γ – interferon gamma; IPC – ischemic preconditioning; HO – haem oxygenase; LPS – Lipopolysaccharide; MCAO – middle cerebral artery occlusion; mitoK_{ATP} – ATP-dependent mitochondrial K channel; MPP - 1-methyl-4-phenylpyridinium; MS – multiple sclerosis; NO – nitric oxide; NOS – nitric oxide synthase; PD – Parkinson disease; PI3K - phosphatidyl inositol 3-kinase; ROS – reactive oxygen species; sGC – soluble guanylyl cyclase; TNF-α - tumour necrosis factor alpha; TNF-γ - tumour necrosis factor gamma

Historical facts

Carbon monoxide (CO) is commonly considered toxic due to 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 carbon monoxide (CO) poisoning (Bernard, 1857). About one century later, CO was also described as cytotoxic for limiting oxidative phosphorylation in cells (*via* cytochrome *c* oxidase inhibition) (Wainio and Greenless, 1960, Savolainen et al., 1980), for further review (Piantadosi, 2002).

Later, CO was recognized as an endogenous molecule in 1949 when Sjöstrand and colleagues identified this gas as a natural metabolite in the exhaled air of healthy humans (Sjostrand, 1949). Nevertheless, it was only in 1968 that CO was published as product of the haem catabolism through haem oxygenase action (Tenhunen et al., 1968). Indeed, this gas is formed by haem-oxygenase (HO) activity following haem degradation, along with free iron and biliverdin (**Figure 1.**).

Insert FIGURE 1 here

In 1988, Harbin and colleagues performed a study about the neurophysiological effect of CO exposure, concluding that acute and low level CO exposure was 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, it was described the first therapeutic action of CO, as vasomodulator (Sammut et al., 1998), as anti-inflammatory (Otterbein et al., 2000) and as anti-apoptotic (Brouard et al., 2000). Since then several distinct applications of CO were explored, namely in organ transplantation, as cardioprotective, anti-inflammatory and anti-apoptotic molecule, and for limiting cell proliferation, in the particular case of atherosclerosis (for review (Motterlini and Otterbein, 2010)). The first applied patent for CO use in Medicine was in 2003 (Yale University. WO03094932A1; 2003), for further information on CO based patents please 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). HO-3 has been found only in the rat brain (McCoubrey et al., 1997), but not in the human. HO is expressed/activated in response to a wide range of different cell stress stimuli, namely oxidative stress, hyperthermia, hypothermia, ischemia, hypoxia, hyperoxia, inflammation or UV (Gozzelino et al., 2010). HO plays a crucial role on the redox state of the cell and is crucial for cellular maintenance and survival in many 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 HO-induced maintenance of both tissue homeostasis and cytoprotection is due to two main actions: (i) the removal of haem group (originated from dying cells or from haemoglobin following haemorrhage) and (ii) to the biological activity of HO products (Grochot-Przeczek et al., 2012). It is worth of note that under stress haem-proteins can release free haem, which in turns become a strong oxidant through Fenton reaction, therefore, catabolism of free haem by HO is crucial for maintaining tissue homeostasis and cytoprotection, for review (Gozzelino et al., 2010).

Haem oxygenase and Central Nervous System

In the brain, basal HO-1 expression is low, while 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, 2004b, Schipper, 2004a), such as in hypoxic-ischemic insult (Sutherland et al., 2009). In several brain pathologies haem oxygenase expression and activity can be involved in the modulation of disease development, as well as in the reestablishment of tissue homeostasis.

HO levels as potential biomarkers in the central nervous system (CNS)

The use of HO protein as a biomarker for brain damage presents some limitations. First, 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, HO increased expression indicates tissue pathological features. Therefore, it is complex to distinguish the HO expression significance, namely whether it qualifies for a pathological biomarker or it represents a predictable value for a favourable outcome.

For example, the levels of HO-1 in the cerebrospinal fluid of infants and children after severe traumatic brain injury are higher (Cousar et al., 2006). Likewise, the levels of HO-1 protein is a promising serum biomarker for early detection of Alzheimer disease , since 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 to an attenuation of oedema formation and a decrease on 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 ischemia lower levels or deletion of HO-1 and -2 expressions are related with a worse outcome (see next

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 haem oxygenase are described in a systematic way.

Cerebrovascular diseases: ischemia and reperfusion

Cerebral ischemia is the main cause of brain damage and the 3rd cause of death in western societies. In adults it is mainly due to stroke, whereas in infants is caused by perinatal complications, in particular 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 ischemia. In humans, there is a long-term increase in the expression of haem oxygenase-1 following focal cerebral infarctions and traumatic brain injury (Beschorner et al., 2000). On the contrary, in a rat model of transient cerebral ischemia, reduction of HO-1 expression is associated with more severe neurodegeneration (Moreira et al., 2007). Protection in ischemic preconditioning (IPC) against permanent ischemic brain injury is dependent on HO-1 expression, since IPC-promoted neuroprotection is abolished in HO-1 gene deleted mice (Zeynalov et al., 2009). Likewise, overexpression of HO-1 by adenovirus vector treatment attenuated brain damage after focal cerebral ischemia in rats (Chao et al., 2013).

Modulation of cerebrovasodilation by haem-oxygenase

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 (Basuroy et al., 2006, Parfenova et al., 2003, Parfenova et al., 2012a, Xi et al., 2011). Moreover, ionotropic glutamate receptors mediate HO activation and endogenous production of CO, which increases cerebral blood flow for maintaining brain homeostasis and neuronal survival during seizures (Parfenova et al., 2012b).

Heme oxygenase and Alzheimer disease

Despite the increasing amount of data demonstrating haem oxygenase as a widespread cytoprotective enzyme, its homeostatic and neuroprotective role in Alzheimer disease (AD) is somewhat controversial. AD is associated with an increase

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 haem oxygenase-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 aging canine model, which develops cognitive dysfunction and neuropathology similar to those in human AD patients, atovastatin-induced up-regulation of HO is 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, since Maes and colleagues have found significantly augmented plasma HO suppressor activity in AD patients relative to healthy elderlies (Maes et al., 2006).

As previously mentioned, HO levels increased in serum of AD patients, being a potential diagnostic biomarker (Mueller et al., 2010). In addition, HO posttranslational modification might also be involved in the development of AD. Barone and colleagues (2012) have 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. Ser-residue phosphorylation and increased oxidative posttranslational modifications of HO-1 were also found in the hippocampus of AD subjects (Barone et al., 2012). Controversially, it was also observed that HO-1 protein levels are lower in post-mortem specimens of cerebrospinal fluid reviewed in (Schipper, 2000).

Thus, HO isoforms and protein posttranslational modifications might also play a role in the debate between neuroprotective vs neurotoxic effects of HO activity in AD.

Heme oxygenase and Parkinson disease

Oxidative stress, accumulation of Lewy bodies and decrease of mitochondrial complex I activity are common features occurring in nigral dopaminergic neurons (DN) during pathological development of Parkinson disease (PD). In *postmortem* human brain specimens collected from PD, immunohistochemistry was used to assess HO-1 expression. In the substantia nigra of both PD and control specimens, moderate HO-1 immunoreactivity was consistently observed in dopaminergic neurons, 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 dopaminergic neurons (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 dopaminergic neurons and reduced the production of tumour necrosis factor alpha (Hung et al., 2008). Using an *in vitro* model of rat midbrain slice culture, in which dopaminergic neurons were induced to die by INF-gamma/LPS treatment, surviving neurons displayed more robust expression of HO-1 whereas treatment with HO-1 inhibitor, zinc protoporphyrin IX, increased cell death levels (Kurauchi et al., 2009). Fibroblast growth factor 9 (FBF9) 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 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 submitted to MPP intraperitoneal 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 and colleagues have suggested the suppression of glial HO-1 activity as a potential therapeutic strategy for treating AD (Schipper et al., 2009). 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 the 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, however normal iron concentration, as the L-ferritin compensates the loss of H-ferritin. Thus, one can also speculate that depending on the ferritin levels and activity, HO could promote cytoprotection or exacerbation of damage. Indeed, Thompson and colleagues have generated a mouse model for AD

and PD, based on a deficiency on H-ferritin, reinforcing the malicious role of iron in neurodegenerative diseases (Thompson et al., 2003). Another important discovery is the existence of mitochondrial ferritin, which is expressed only in 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, HO effect on neurodegenerative diseases must be studied conjointly with ferritin activity.

Neuroinflammation and multiple sclerosis

During the last decade, several data in the literature 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 through increasing expression of HO-1.

Multiple sclerosis (MS) is an autoimmune disease affecting CNS with inflammatory lesions, demyelination and axonal loss (Fagone et al., 2012). In 2001 it was first shown the protective and anti-inflammatory role of HO-1 activity in an experimental model of multiple sclerosis, the experimental autoimmune encephalomyelitis (EAE). Pharmacological induction of HO-1 with haemin effectively inhibited EAE, while 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, multiple sclerosis 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 are also some works stating that over-expression 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 reverted by the addition of iron chelator deferoxamine (Song et al., 2006). Likewise, in astrocytes of spinal cord from MS patient expressed higher levels of HO-1 than astrocytes from control subjects (Mehindate et al., 2001).

Pain

Carvalho and colleagues (2011) 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 sGC inhibitor have shown that the anti-nociceptive action is reduced whenever HO activity is prevented, being this effect dependent on sGC (Carvalho et al., 2011).

Heme oxygenase 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, red wine, etc. 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 firstly 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 cell culture of neurons and astrocytes curcumin protects against inflammation, oxidative damage and cell death (Table 1). Ginkgo biloba, which is an extract used in traditional Chinese medicine, has been wildly described as neuroprotective compound. In Table 1 there are several examples showing HO implication in Ginkgo biloba-induced neuroprotection using *in vitro* and *in vivo* models. Resveratrol, which is a component of red wine associated to cardioprotection and neuroprotection, was demonstrated to confer its healthy properties by HO-1 activation *in vitro* and *in vivo* (Sakata et al., 2010, Zhuang et al., 2003a), (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 last two decades, many CO biological functions have been described and a great effort is under progress for developing its use in 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 partially, delivered in the body through blood plasma flow and carboxyhaemoglobin, leading 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 synthesising molecules able to deliver CO, which were first denominated as carbon monoxide releasing molecules – CORMs (Motterlini et al., 2002). Although an enormous number of CORMs was 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 issued must be overcame in the development of CORMs, namely: water-insolubility, toxic chemical structures, promotion of high levels of carboxyhaemoglobin, chemical instability, etc; for further review on the their development (Romao et al., 2012). In the particular case of central nervous system, the most studied pro-drugs were CORM-A1, CORM-2 and CORM-3. CORM-A1 ($[H_3BCO_2]Na_2$) is a boranocarbonate molecule; while the transition metal based molecules are: CORM-2 $[Ru(CO)_3Cl_2]_2$, which is a dimer and insoluble in water; and the water soluble CORM-3 ($[Ru(CO)_3Cl(\kappa^2\text{-}H_2NCH_2CO_2)]$). 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). Still, 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. BBB isolates the brain and decreases the risk of infection and the entrance of toxins. Despite the amount of research work done about CORM and the brain, it is not fully clarified the ability of any CORM to cross the BBB; while it is accepted that CO gas can cross biological membranes.

CO and Central Nervous System

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

Cerebrovascular disease

Low levels of inhaled CO were beneficial against cerebral hypoxic and ischemic insult in experimental rodent models. In mice, CO exposure at 250 ppm for 18h immediately after permanent middle cerebral artery occlusion (MCAO) decreased by about 30% the infarct volume, after 7 days (Wang et al., 2011). Likewise, in a transient MCAO model (90 minutes of focal ischemia followed by 48h of reperfusion) inhalation of 125 ppm of CO immediately at the onset of reperfusion also decreased about 30% the brain damage after 48h. While, inhalation of CO at 250 ppm in the same model and conditions decreased around 60% the brain damage. Interestingly, when the CO inhalation is performed 1h or 3h 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, whenever CORM-3 is administered 5 minutes before or 3 days after the intracerebral haemorrhage stimulus (injection of collagenase), decreased the inflammatory response. The opposite effect is achieved when CORM-3 is injected 3h after the haemorrhagic insult (Yabluchanskiy et al., 2012). Thus, the time window for CO administration is crucial for its biological functions and further studies are urgently necessary. In a perinatal rat model of cerebral hypoxia-ischemia, CO exposure at 250 ppm for 1h/day on the 3 days prior to the ischemic insult decreased 64% the apoptotic cell death in the hippocampus (Queiroga et al., 2012b). In another perinatal experimental system, a piglet model of deep hypothermic circulatory arrest (DHCA), mimicking the open-heart surgeries procedures, inhalation of 280 ppm CO, for 3h, 1 day prior to surgery limited cell

death in the neocortex and hippocampus (Mahan et al., 2012). In the in vivo retinal ganglion cells model of ischemia and reperfusion, CO gas preconditioning (Biermann et al., 2010) and postconditioning (Schallner et al., 2012) also promoted neuroprotection. Finally, CORM-A1 (2 mg/kg, intraperitoneally) administration 30 minutes before seizure chemical induction protects 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, experimental autoimmune encephalomyelitis (EAE) in SJL mice, a prolonged prophylactic treatment with CORM-A1 reduces the incidence of the disease and attenuates the inflammatory infiltrations of the spinal cords (Fagone et al., 2011). Exogenous CO administration (250 ppm) suppressed myelin-reactive immune cells 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 have demonstrated that treating mice with CORM-2 and CORM-3 for 10 to 20 days following sciatic nerve injury improved the local anti-nociceptive 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 over-expression (Hervera et al., 2012, Hervera et al., 2013b, Hervera et al., 2013a).

Brain cells and carbon monoxide: in vitro *approaches*

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

Neuroprotection is not an exclusive matter of neuronal functioning. Indeed, one must also target glial cells for promoting neuroprotection. The physiological role of astrocyte, microglia, oligodendrocytes and endothelial cells is the maintenance of brain homeostasis, metabolism and neuronal functioning. Therefore, modulation of glial cells functioning is crucial for promoting neuroprotection. Likewise, regulation of astrocytic metabolism and prevention of astrocytic apoptosis against oxidative stress is decisive for the brain homeostasis maintenance. Indeed, CO gas limited

astrocytic apoptosis by two distinct ways: (i) direct prevention of mitochondrial membrane permeabilisation and the consequent release into the cytosol of pro-apoptotic factors (Queiroga et al., 2010) and (ii) improvement of cellular metabolism and increase on oxidative phosphorylation and mitochondrial population (Almeida et al., 2012).

Furthermore, excessive inflammation response can be detrimental, being CO's modulation of inflammation in microglia very important for the control of neuroinflammation. Many studies of the CO anti-inflammatory effect have been done *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 INF-γ stimulus. It was described that inhibition of mitogen-activated protein kinases phosphatidyl inositol 3-kinase (PI3K) exacerbated the anti-inflammatory effect of CORM-3. On the opposite, sGC, NOS and HO activity had no influence on CORM-3 mode of action (Bani-Hani et al., 2006b, Bani-Hani et al., 2006a). Taken all together, the ability of CO in limiting 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 blood brain barrier (BBB). Parfenova and colleagues have demonstrated that in cerebral microvascular endothelial cells (CMEC) present HO-1 and HO-2 isoforms and their endogenous CO regulates vascular tone in response to glutamate (Parfenova et al., 2001, Parfenova et al., 2003, Leffler et al., 2011). Likewise, endogenous and exogenous CO prevents endothelial cell death *via* modulation of Nox4 NADPH activity (Basuroy et al., 2009, Basuroy et al., 2011), see next section. Finally, CORM-A1 prevents blood brain barrier dysfunction by limiting glutamate-induced apoptosis and oxidative stress in CMEC (Basuroy et al., 2013). Still, one can speculate 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 in the literature to contribute to the cellular and biochemical mechanisms associated with CO's biological role. Yet, those biochemical pathways and the actual physiological target(s) of CO are still a matter of great 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), which modulate 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 both Fe^{2+} and Fe^{3+} (Boczkowski et al., 2006).

In CNS, the pathways and potential targets of CO are still poorly understood with few available data on the literature concerning the mechanisms by which CO confers neuroprotection, anti-neuroinflammation or vasomodulation, being urgent the development of more scientific research on this subject. This section focuses and discusses the existing data about CO's pathways in the brain.

Soluble Guanylyl Cyclase (sGC) and Nitric Oxide Synthase (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, since high concentrations of CO are usually required for activating sGC. On the contrary, much lower NO levels are needed for activating sGC. Regarding neuronal cells, activation of sGC and NOS and the respective production of cyclic guanosine monophosphate (cGMP) and NO, were shown to be important for CO-induced neuroprotection against excitotoxicity and ischemic insult (Vieira et al., 2008, Schallner et al., 2013). In a model of permanent ischemic 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., 2006b, Bani-Hani et al., 2006a). Still, increased levels of cGMP appeared to be downstream to endogenous CO production in astrocytes (Imuta et al., 2007); while in cerebral microvessels cGMP signalling appeared to be upstream of CO modulation, since glutamate-induced NOS activation leaded 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 (MOR), δ-opioid receptors (DOR) and cannabinoid-2 receptor (CB2R) are improved by CO (CORM-2 and CORM-3) in a NO-dependent fashion during chronic inflammatory and neuropathic pain (Hervera et al., 2013a, Hervera et al., 2013b).

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 (for review (Bilban et al., 2008, Queiroga et al., 2012a). At least two cellular proteins are recognized to be 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 CO-cytotoxic effects; actually it is widely accepted that 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 on cytochrome *c* oxidase activity, while after 30 minutes (and up to 24h) 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 promotes ROS generation (Queiroga 2010); and the use of β-carotene for limiting ROS levels has prevented the anti-apoptotic effect of CO in astrocytes, as well as the CO-induced protection against mitochondrial membrane permeabilisation (Queiroga et al., 2010). In primary culture of cerebellar granular neurons, small amounts of ROS are produced upon CO treatment; and whenever ROS generation is prevented by butyl-hydroxytoluene, the neuroprotective effect of CO is reverted, indicating the essential role of ROS as signalling factors (Vieira et al., 2008).

In inflammatory brain diseases, NADPH oxidase, in particular its major isoform Nox4, generates ROS, which can initiate both death and survival pathways in TNF-α-challenged cerebral microvascular endothelial cells (CMVEC). Endogenous and exogenous CO limits the production of anion superoxide by Nox4 NADPH, preventing endothelial cell death caused by TNF-alpha-induced oxidative stress (Basuroy et al., 2009). While, Nox4 NADPH-derived reactive oxygen species (ROS) also initiate 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, whereas NADPH oxidase produces ROS that increase CO generation, which, in turn, prevents NADPH oxidase activity, its excessive anion superoxide production and oxidative stress.

In no-brain systems there are other potential pathways for biological CO action related to ROS signalling and mitochondria. In cardiomyocytes CO-induced mitochondrial ROS production may control of mitochondrial biogenesis leading to cytoprotection (Suliman et al., 2007a, Suliman et al., 2007b). 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 CO's target be since inhibition of complex II (malonate addition) reverted the CO-induced augmentation of oxygen consumption and the uncoupling effect (Lo Iacono et al., 2011). In contrast, in liver system, CO has been described as 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 hepatotoxicity (Gong et al., 2004). Based on 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 possess 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 calcium-sensitive potassium (BK) 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 calcium-dependent potassium channels (Jaggar et al., 2005). Namely, astrocytic HO-2-derived CO causes glutamatergic dilation of pial arterioles, by activating smooth muscle cell large-conductance Ca(2+)-activated K(+) (BK(Ca)) channels (Leffler et al., 2011). Thus, one can speculate that CO binds directly to BK(Ca) channel-bound haem for controlling dilation and constriction of vasculature (Leffler et al., 2011).

Insert FIGURE 2 here

Future challenges for CO administration

The first issue for the clinical potential use of CO in cerebral pathologies is the lack of information about its mode of action. Despite the described different mechanisms about the cellular and biochemical pathways of CO, the precise underlying signalling mechanisms and the exact molecular target(s) of CO are poorly elucidated. It is worth of note that elucidating the potential protein targets of CO under physiological conditions is extremely complex, since 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 complexity to study CO targets under physiological conditions. Based on the fact that CO seems to mimic preconditioning, promoting a tissue tolerance state, one might explore the classical activator and transducer factors involved in preconditioning and CO. For instance, preconditioning stimulus leads to upregulation of vascular endothelial growth factor (VEGF) (Laudenbach et al., 2007, Wick et al., 2002), activation of hypoxic inducible factor (HIF-1) (Chu et al., 2010, Ratan et al., 2004) or expression of erythropoietin (Ruscher et al., 2002), being these factors promising candidates for CO related pathways. Indeed, in macrophages CO has been described to stabilize HIF-1 (Chin et al., 2007).

The second challenge concerning CO administration is achieving the best way to specifically deliver CO in the target tissue, avoiding high concentrations of

carboxyhaemoglobin. Many studies have been performed for developing CORMs avoiding systemic toxicity related to carboxyhaemoglobin, for further review (Romao et al., 2012, Zobi, 2013). Indeed, there are CORMs with different effects on carboxyhaemoglobin; while CO gas exposure and CORM-A1 administration reach similar levels of carboxyhaemoglobin (Otterbein et al., 1999, Ryan et al., 2006), very low changes in carboxyhaemoglobin levels were observed in the case of CORM-3 (Guo et al., 2004). Nevertheless, how and where administrate CORMs to deliver CO is still a matter of intensive research. Likewise, chemical modifications of CORMs are under progress to target these molecules to a specific organ/cell type (Fagone et al., 2012). Up to date the best example concerns ALF 794 specifically targeting liver against acute injury (Marques et al., 2012). Still, several questions remain unanswered: How CORM is transported in the blood flow? Does CORM bind to any protein present in the blood for maintaining its stability? Does CORM need to cross the cellular plasma membrane? Is CO delivered in the extracellular space, getting intracellularly by membrane diffusion? Therefore, further studies are necessary for disclosing 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 gasotransmitter as novel drug for medical applications.

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

The time window for CO administration is essential for the best outcome and depends on the pathophysiological situation. Preconditioning is one of the claimed CO-induced processes, where CO stimulates endogenous cellular pathways of protection (anti-inflammatory, anti-apoptotic, pro-survival, pro-homeostatic, etc) (Bilban et al., 2008, Queiroga et al., 2012b, Piantadosi et al., 2008). In this case, the therapeutic strategy consists of CO administration previously to the injury; for instance in high risk patients of developing cerebral ischemia (before great cardiac surgeries, high risk newborn infants, aging patients with cardiovascular complications and risk of ischemic stroke). Still, during the development of chronic diseases, exogenous CO can be used as a periconditioning agent, namely Alzheimer disease, Parkinson disease or multiple sclerosis (Fagone et al., 2011). Another evidence in the literature concerns the ability of CO to respond against acute injury, thus CO can be applied after injury, as postconditioning strategy, such as described in cerebral ischemia, intracerebral haemorrhage and seizures (Wang et al., 2011, Yabluchanskiy et al., 2012, Zeynalov and Dore, 2009).

Final conclusions

There is a groundbreaking evidence supporting the protective role of carbon monoxide (and haem oxygenase) in the central nervous system in the context of several pathologies: cerebrovascular diseases, neuroinflammation, multiple sclerosis, pain, Alzheimer and Parkinson diseases.

The therapeutic advent leads to the need of further development of CO sources, others from CO gas, to overcome the carboxyhaemoglobin toxicity. CORMs have been increasingly used with successful and interesting results. Nevertheless, it was the inhaled CO that has been firstly proven to be safe and tolerable in humans.

Independently of the administration route and regardless of the cell type, CO appears to modulate several cellular players, such as cytochrome *c* oxidase, nitric oxide synthase (NOS), soluble guanylyl cyclase (sGC) or NADPH oxidase; ROS signaling and mitochondria targeting are also involved in CO's pathways. Nevertheless, further research is urgently necessary for precisely clarify the biological CO target(s) and pathways.

In conclusion, carbon monoxide has travelled far away from being an invisible enemy, becoming a possible consistent therapeutic solution.

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Statement of conflict of interest

There is no conflict of interest.

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