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Carbon monoxide and anesthesia-induced neurotoxicity[☆]

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Abstract

The majority of commonly used anesthetic agents induce widespread neuronal degeneration in the developing mammalian brain. Downstream, the process appears to involve activation of the oxidative stress-associated mitochondrial apoptosis pathway. Targeting this pathway could result in prevention of anesthetic toxicity in the immature brain. Carbon monoxide (CO) is a gas that exerts biological activity in the developing brain and low dose exposures have the potential to provide neuroprotection. In recent work, low concentration CO exposures limited isoflurane-induced neuronal apoptosis in a dose-dependent manner in newborn mice and modulated oxidative stress within forebrain mitochondria. Because infants and children are routinely exposed to low levels of CO during low-flow general endotracheal anesthesia, such anti-oxidant and pro-survival cellular effects are clinically relevant. Here we provide an overview of anesthesia-related CO exposure, discuss the biological activity of low concentration CO, detail the effects of CO in the brain during development, and provide evidence for CO-mediated inhibition of anesthesia-induced neurotoxicity.

Keywords

Carbon monoxide; Exposure; Low-flow anesthesia; General anesthesia; Anesthesia-induced neurotoxicity; Cytoprotection; Therapy; Exogenous; Endogenous; Apoptosis; Oxidative stress; Mitochondria

1. Introduction

The majority of commonly used anesthetic agents induce widespread neuronal degeneration in the developing mammalian brain, resulting in behavioral impairments and cognitive deficits later in life (Jevtovic-Todorovic et al., 2003; Stefovskaja et al., 2008; Istaphanous and Loepke, 2009; Brambrink et al., 2010; Istaphanous et al., 2011). This toxicological effect has been demonstrated in a variety of newborn animal models including non-human primates (Brambrink et al., 2010; Olney et al., 2004; Rizzi et al., 2010). Although a causal relationship has yet to be established in humans, several retrospective studies have demonstrated an association between anesthesia exposure in young children and subsequent

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defects in learning and scholastic performance (Wilder et al., 2009; DiMaggio et al., 2009; Flick et al., 2011; Psaty et al., 2015). The exact mechanisms of anesthesia-induced neurotoxicity have not been fully elucidated, however, the downstream process appears to involve activation of the oxidative stress-associated mitochondrial apoptosis pathway (Olney et al., 2004; Yon et al., 2005; Bai et al., 2013; Boscolo et al., 2013; Zhang et al., 2010). Thus, targeting this pathway is a strategy that could lead to development of novel therapeutic agents which will prevent or limit anesthetic toxicity in the immature brain.

Carbon monoxide (CO) is a colorless, odorless, and tasteless gas that is biologically active in many different tissues and organs (Iqbal et al., 2012; Kao and Nañagas, 2005). In the brain, CO has the potential to cause neurotoxicity or provide neuroprotection depending on the context, duration, and concentration of exposure. Overt toxicity is the most widely recognized effect of CO due to the well-characterized tissue hypoxia that results following exposure to high concentrations (Kao and Nañagas, 2005). However, at low concentrations, CO acts as a signaling molecule, affecting several different cellular pathways in a more intricate and complex manner (Kapetanaki et al., 2009; Ignarro et al., 1982; Furchgott and Jothianandan, 1991; Morita et al., 1997; Kim et al., 2006; Otterbein et al., 2000; Kim et al., 2005a; Kim et al., 2005b; Rhodes et al., 2009; Lee et al., 2011; Chiang et al., 2013). These sub-toxic concentrations have been shown to confer cytoprotection through an array of mechanisms (Kapetanaki et al., 2009; Ignarro et al., 1982; Furchgott and Jothianandan, 1991; Morita et al., 1997; Kim et al., 2006; Otterbein et al., 2000; Kim et al., 2005a; Kim et al., 2005b; Rhodes et al., 2009; Lee et al., 2011; Chiang et al., 2013). As a result, low dose CO is currently being explored as a novel treatment for a variety of diseases processes.

Recently, we demonstrated that exposure to low concentration CO limited isoflurane-induced neuronal apoptosis in a dose-dependent manner and modulated oxidative stress within forebrain mitochondria in a newborn mouse model of anesthesia-induced neurotoxicity (Cheng and Levy, 2014; Cheng et al., 2015). Because infants and children are routinely exposed to low levels of CO during low-flow general endotracheal anesthesia, investigation of CO as a potential therapy to offset the deleterious effects of anesthetics in the developing brain has clinical relevance. Here we provide an overview of anesthesia-related CO exposure, discuss the biological activity of low concentration CO, detail the effects of CO in the brain during development, and provide evidence for CO-mediated inhibition of anesthesia-induced neurotoxicity.

2. CO exposure during low-flow general endotracheal anesthesia

Low-flow anesthesia (LFA) is a commonly used, cost-saving paradigm which permits rebreathing in order to conserve volatile anesthetic agents (Baum and Aitkenhead, 1995). Although there is no widely accepted definition, LFA generally refers to an anesthetic technique involving delivery of fresh gas at 1 l per minute or less in adults (Nunn, 2008). In children, a low-flow approach results in rebreathing when the rate of minute ventilation exceeds the rate of fresh gas flow (FGF) (Levy et al., 2010; Nasr et al., 2010). Exposure to CO routinely occurs during LFA and the extent of exposure inversely correlates with the FGF-to-minute ventilation ratio (Levy et al., 2010; Tang et al., 2001). Infants and children have been shown to inspire up to 20 ppm (ppm) CO during a low-flow general endotracheal

anesthetic when the rate of FGF was set below the rate of minute ventilation (Levy et al., 2010; Nasr et al., 2010). CO manifests in the anesthesia breathing system in this setting from endogenous patient sources but may also be generated exogenously within the circuit (Coppens et al., 2006; Woehlck, 2001). Exogenous CO is produced when volatile anesthetic agents are degraded by conventional carbon dioxide absorbents while endogenously formed CO is present in exhaled breath (Coppens et al., 2006; Woehlck, 2001; Baxter et al., 1998).

Degradation occurs with all of the volatile anesthetics currently in use today and is catalyzed by the base of conventional absorbents (Baxter et al., 1998; Fang et al., 1995; Keijzer et al., 2005; Woehlck et al., 2001). Strong alkali hydroxides, such as potassium and sodium hydroxide, are the chemical components that are primarily responsible (Neumann et al., 1999). Water content is another key factor that determines the degree of anesthetic degradation and the amount of CO formed is inversely proportional to the amount of water in the absorbent (Fang et al., 1995; Baxter and Kharasch, 1997). Other important variables that influence anesthetic degradation include the absorbent temperature, type of volatile agent, anesthetic concentration, and patient carbon dioxide production (Fang et al., 1995; Fan et al., 2008).

In order to limit chemical breakdown of volatile anesthetics and CO formation, the Anesthesia Patient Safety Foundation (APSF) recommends using absorbents that lack strong alkali hydroxides or avoiding conventional absorbent desiccation (Olympio, 2016; Murray et al., 1999). Although adherence to the APSF guidelines reduces the risk of generating overtly toxic levels of CO, it does not prevent exposure to low concentrations of CO. One reason for this is that even fully hydrated conventional absorbents are capable of degrading volatile agents (Fan et al., 2008). For example, completely hydrated soda lime has been shown to generate up to 23 ppm CO during a 2-h desflurane anesthetic (Fan et al., 2008). Although the amount of CO produced from hydrated absorbent is markedly less than that generated by dried absorbent, CO formation still occurs and varies based on the rate of FGF (Fan et al., 2008). This is because smaller volumes of fresh gas limit the dilution of CO within the breathing circuit, thus, lower rates of FGF result in higher CO concentrations (Fan et al., 2008).

Another source of CO exposure during LFA is patient-derived, endogenously generated CO (Woehlck, 2001). CO is formed naturally within the liver, spleen, and kidney, and within the central nervous system and reticuloendothelial system during heme catabolism (Hayashi et al., 2004). Following its generation, CO diffuses into the circulation, binds to hemoglobin, and is excreted by the lungs as a component of exhaled breath (Hayashi et al., 2004). During LFA, exhaled CO is not scavenged or removed from the closed anesthesia breathing circuit (Woehlck, 2001). As a consequence, patients rebreathe exhaled CO, resulting in an active exposure (Woehlck, 2001).

Therefore, patients are commonly exposed to CO during low-flow general endotracheal anesthesia. In the current era, the majority of anesthesia-related CO exposures are sub-toxic (below the threshold for tissue hypoxia). Although the significance of such exposures is not well understood, it is known that CO acts as a signaling molecule at these levels. Low concentration CO exerts biological activity in many organs and tissues including the

immature brain (Levy, 2015). Thus, CO exposure during LFA could have implications for infants and children. In the next few sections we will review the cellular effects of low concentration CO and detail the known neurodevelopmental consequences of such exposures.

3. Biological activity of CO

Following inhalation, CO readily diffuses across the alveolar capillary membrane to bind to hemoglobin, forming carboxyhemoglobin (COHb) (Smithline et al., 2003). The affinity of hemoglobin is 240 times greater for CO than for oxygen and high COHb levels can interfere with oxygen binding and dissociation (Hauck and Neuberger, 1984; Gorman et al., 2003). This leads to impaired tissue oxygen delivery by shifting the oxygen-hemoglobin dissociation curve to the left (Gorman et al., 2003). High concentrations of CO can also directly interfere with aerobic cellular energy production by binding to hemoproteins within the cytosol and mitochondria (Brown and Piantadosi, 1990; Iheagwara et al., 1972). Thus, toxic CO exposures result in tissue and cellular hypoxia and clinically detectable signs and symptoms manifest when COHb levels exceed 20% (Table 1) (Kao and Nañagas, 2005).

The amount of COHb formed and the manifestation of toxic effects relate to the concentration and the duration of CO exposure (Table 1) (Winter and Miller, 1976; Raub, 1999). This time-weighted relationship dictates the degree of toxicity, or lack thereof (Winter and Miller, 1976). For example, exposure to between 70 and 120 ppm CO for approximately 4 h is considered a low concentration exposure and results in COHb levels between 10 and 20% (Winter and Miller, 1976; Raub and Benignus, 2002; Tomaszewski, 2002). Such an exposure is usually asymptomatic, does not cause tissue hypoxia, and is not life threatening (Winter and Miller, 1976; Raub and Benignus, 2002; Tomaszewski, 2002). Lack of detectable signs and symptoms defines this type of CO exposure as sub-clinical and sub-toxic (Winter and Miller, 1976). On the other hand, exposure to CO concentrations in excess of 200 ppm results in COHb levels of approximately 30% and readily causes headache, dizziness, and impaired judgment (Winter and Miller, 1976). While, inspiring >800 ppm CO is considered a high concentration exposure and results in COHb levels that exceed 60% and can rapidly lead to seizures, coma, and death (Winter and Miller, 1976).

In contrast to the hypoxia-inducing, toxic concentrations, low dose CO exerts complex biological activity in a variety of cells and tissues even at nanomolar concentrations (Kapetanaki et al., 2009). These gasotransmitter properties were first uncovered following discovery of the ability of CO to weakly stimulate soluble guanylate cyclase to generate cyclic guanosine 3',5'-monophosphate (cGMP) (Ignarro et al., 1982; Furchgott and Jothianandan, 1991; Morita et al., 1997). Since its identification as a signaling molecule, CO has been shown to modulate several p38 mitogen-activated protein kinase (MAPK)-related signaling pathways via both cGMP-dependent and independent processes, directly activate calcium-dependent potassium channels, induce protein kinase B (Akt) phosphorylation via the phosphatidylinositol 3-kinase/Akt pathway, and regulate the activity of a variety of hemoproteins by binding to the iron center within their heme prosthetic groups (Kim et al., 2006; Bauer and Pannen, 2009).

Examples of CO-mediated cGMP-dependent activity include inhibition of smooth muscle cell proliferation and platelet aggregation, neurotransmission, and vasodilation, while CO-mediated cGMP-independent effects include anti-inflammation, anti-apoptosis, and anti-proliferation (Otterbein et al., 2000; Kim et al., 2005a; Kim et al., 2005b). In addition, low concentrations of CO have been shown to promote host survival and confer cytoprotection by stimulating mitochondrial biogenesis, inducing autophagy, and accelerating the resolution of inflammation (Rhodes et al., 2009; Lee et al., 2011; Chiang et al., 2013). Thus, it is through many different pathways that low concentration CO has shown therapeutic potential (Kim et al., 2006; Bauer and Pannen, 2009).

The biological activity of low dose CO has been shown to attenuate injury and protect different tissues and cell types in a variety of clinically-relevant settings (Otterbein et al., 1999; Dolinay et al., 2004; Fredenburgh et al., 2015; Nemzek et al., 2008; Bathoorn et al., 2007; Fujita et al., 2001; Zhang et al., 2005; Kohmoto et al., 2007; Mishra et al., 2006; Song et al., 2003; Zhang et al., 2003; Boutros et al., 2007; Goebel et al., 2008; Fujimoto et al., 2004; Lavitrano et al., 2004; Sato et al., 2001; Akamatsu et al., 2004; Nakao et al., 2006; Vieira et al., 2008; Mahan et al., 2012; Queiroga et al., 2012; Wang et al., 2011). A summary of the various preclinical and clinical studies that have demonstrated such cytoprotective effects of low concentration CO is presented in Table 2. Importantly, the brain and neuronal cell populations have been shown to be therapeutic targets of low concentration CO. For example, preconditioning cultured mouse neurons with 250 ppm CO enhanced survival and protected cells from apoptotic-induced death (Vieira et al., 2008). In other work, exposing piglets to 250 ppm CO for 3 h, 1 day prior to CPB with deep hypothermic circulatory arrest, completely prevented cell death in the neocortex and hippocampus (Mahan et al., 2012). Furthermore, preconditioning newborn mice with 250 ppm CO protected cells within the hippocampus from hypoxia- and ischemia-induced apoptosis (Queiroga et al., 2012). In a study that explored the post-conditioning potential of CO, exposure to 250 ppm CO for 18 h following complete occlusion of the middle cerebral artery in mice, reduced infarct size by about 30% (Wang et al., 2011). Thus, low concentration CO has the potential for neuroprotection.

The developing brain is unique in that it is sensitive to a variety of biologically active agents during critical periods of development (Grandjean and Landrigan, 2006; Rice and Barone, 2000). The neurodevelopmental consequences of this vulnerability relate to the timing of exposure and the specific developmental processes that are impacted by the various agents (Rice and Barone, 2000). CO has the potential to act as a neurotoxin or neuroprotectant in the immature brain depending on the context, duration, and concentration of exposure. In the next section, we will detail the known neurodevelopmental effects of low concentration CO exposure during development.

4. Neurodevelopmental effects of low concentration CO exposure

Coordination of proliferation, differentiation, migration, synaptogenesis, apoptosis, and myelination is critical for normal brain development (Rice and Barone, 2000). Interruption of any of these developmental processes can result in functional neurologic impairment (Rice and Barone, 2000). Because the human brain begins to develop in utero and continues

to mature postnatally, vulnerability to various biological agents starts in the fetal period and extends for years into childhood (Grandjean and Landrigan, 2006). This neurodevelopmental susceptibility relates to the fact that infants and children can rapidly absorb toxins and have reduced ability to detoxify them (Grandjean and Landrigan, 2006).

Although much is known about the overt toxicity of exposure to high concentrations of CO, little is understood about the neurologic effects of sub-toxic CO exposure. However, perinatal low concentration CO exposure has been shown to interrupt many of the developmental processes that are critical for normal brain maturation. Neuronal proliferation and differentiation, myelination, and natural neuronal apoptosis have all been shown to be adversely impacted by sub-clinical CO exposures (Benagiano et al., 2005; Carratù et al., 2000; Fechter et al., 1987; Fechter, 1987).

For example, perinatal exposure to 75, 150, or 300 ppm CO, beginning early in gestation, resulted in a dose-dependent decrease in cerebellar weight and the number of γ -aminobutyric acid (GABA)-ergic neurons in the cerebellum of exposed rat pups (Fechter et al., 1987). According to the investigators, these findings indicated CO-mediated disruption of neuronal proliferation in utero (Fechter et al., 1987). Such CO exposures also led to a significant increase in deoxyribonucleic acid (DNA) content and cell number in the neostriatum likely due to enhanced glial proliferation in response to neuronal loss (Fechter, 1987). With regard to differentiation, prenatal exposure to 75 ppm CO reduced the number of glutamic acid decarboxylase/GABA positive neuronal bodies and axon terminals in the cerebellar cortex in rats later in life (Benagiano et al., 2005). As for myelination, rats exposed to either 75 or 150 ppm CO during their entire gestational period demonstrated significantly decreased sciatic nerve myelin sheath thickness as adults (Carratù et al., 2000). Although CO has been shown to impair proliferation, differentiation, and myelination during development, the exact mechanisms remain unknown. Because resultant COHb levels in these models of CO exposure were well below the threshold necessary to impair oxygen delivery, CO-mediated inhibition of these processes was not the result of tissue hypoxia.

With regard to developmental apoptosis, postnatal exposure to 5 ppm or 100 ppm CO for 3 h impaired cytochrome *c* release from forebrain mitochondria, decreased caspase-3 activity, reduced the number of activated caspase-3 positive cells in the neocortex and hippocampus, and decreased forebrain apoptosis in a dose-dependent manner in 10 day old mouse pups (Cheng et al., 2012). CO-mediated inhibition of programmed cell death in this work resulted from a concentration-dependent inhibition of the peroxidase activity of cytochrome *c* (Cheng et al., 2012). Peroxidation of cardiolipin by cytochrome *c* permits mobilization of cytochrome *c* from the inner mitochondrial membrane; a prerequisite for its release into the cytosol upon activation of the intrinsic apoptosis pathway (Fig. 1) (Cheng et al., 2012). Nanomolar concentrations of CO can bind to the cytochrome *c*-cardiolipin complex within mitochondria and inhibit the peroxidase activity of cytochrome *c* (Kapetanaki et al., 2009). Thus, the findings suggest one potential mechanism for the anti-apoptotic effects of low concentration CO in the developing brain (Cheng et al., 2012). The postnatal phase of natural programmed cell death is important for selective elimination of aberrant and excess neurons (Chan et al., 2002). Consequently, CO-mediated inhibition of apoptosis in the newborn mouse brain impaired neuron elimination during this developmental window as

evidenced by increased neuron specific antigen content, greater number of neurons, and megalencephaly one week after exposure (Cheng et al., 2012).

A number of experimental investigations have demonstrated that perinatal exposure to low concentration CO impairs memory, learning, and behavior later in life (De Salvia et al., 1995; Fechter and Annau, 1980; Giustino et al., 1999). For example, maternal exposure to 75 or 150 ppm CO during pregnancy led to abnormal habituation and working memory in prenatally exposed juvenile male rats while sparing motor activity (Giustino et al., 1999). In addition, exposure to 150 ppm CO during gestation resulted in permanently impaired acquisition of a two-way active avoidance task in male rats tested at 3 months of age (De Salvia et al., 1995). Postnatal exposure to low concentration CO has also been shown to impact rodent neurodevelopment (Cheng et al., 2012). For example, 3-h exposure to 5 ppm or 100 ppm CO in 10 day old mice impaired reference memory, memory retention, and spatial working memory in a dose-dependent manner several weeks after exposure and resulted in significant avoidance activity, indicating abnormal socialization (Cheng et al., 2012).

The experimental evidence suggests that perinatal exposure to low concentrations of CO can disrupt normal neurodevelopment. These findings raise concern about the safety of such exposures in infants and children. As mentioned previously, levels of CO encountered during a general anesthetic fall largely within the sub-clinical, low concentration range. However, due to a paucity of research, the consequences of such anesthesia-related exposures in the pediatric population are unknown. Alternatively, despite the vulnerability of the immature nervous system to low concentration CO, the potential exists for CO to paradoxically provide neuroprotection in certain clinical settings or when combined with other neurotoxins. For example, it is feasible that the pro-survival, anti-apoptotic effects of sub-clinical CO could limit and offset the pro-apoptotic and harmful effects of anesthetics in the developing brain. Given that low concentration CO exposure occurs commonly during LFA, we investigated the neurobiological effects of combined exposure to sub-clinical CO with isoflurane in newborn mice. In the next section, we will review the evidence for CO-mediated inhibition of anesthesia-induced neurotoxicity in this model. The findings highlight the potential for low dose CO to be developed as a novel therapy to target anesthesia-mediated activation of the oxidative stress-associated mitochondrial apoptosis pathway in the immature brain.

5. CO-mediated inhibition of anesthesia-induced neurotoxicity

The majority of commonly used anesthetic agents cause neuronal degeneration in many regions of the developing mammalian brain (Jevtovic-Todorovic et al., 2003; Stefovaska et al., 2008; Istaphanous and Loepke, 2009; Brambrink et al., 2010; Istaphanous et al., 2011). The period of maximum vulnerability coincides with the peak of synaptogenesis and anesthesia-induced neurotoxicity has been shown to result in significant neuron loss, behavioral impairments, and cognitive deficits in many different newborn animal models (Brambrink et al., 2010; Olney et al., 2004; Rizzi et al., 2010). The exact mechanisms underlying anesthesia-induced neurotoxicity are not completely understood, however, there is evidence that the downstream process may be mediated by the oxidative stress-associated

mitochondrial pathway of apoptosis (Olney et al., 2004; Yon et al., 2005; Bai et al., 2013; Boscolo et al., 2013; Zhang et al., 2010).

Anesthetic agents cause oxidative stress by inducing reactive oxygen species (ROS) formation within neurons during exposure (Bai et al., 2013; Zhang et al., 2010). ROS produced in this setting have been shown to arise from mitochondrial sources and accumulate within mitochondria (Bai et al., 2013; Zhang et al., 2010). Superoxide, the principal free radical generated within mitochondria, is known to be produced by complexes I and III of the electron transport chain (Fig. 1) (Srinivasan and Avadhani, 2012; Srinivasan and Avadhani, 2012). Following their generation, superoxide radicals are converted to hydrogen peroxide (H_2O_2) by superoxide dismutase or via spontaneous dismutation (Bai et al., 2013). In the presence of H_2O_2 , cytochrome *c*, the mobile electron carrier that normally transfers electrons between complex III and cytochrome oxidase (COX) during oxidative phosphorylation, exerts peroxidase activity and oxidizes cardiolipin to hydroperoxycardiolipin (Fig. 1) (Kapetanaki et al., 2009). This permits cytochrome *c* to become unbound from cardiolipin and to mobilize from the inner mitochondrial membrane (Kapetanaki et al., 2009). Following anesthetic-mediated GABA_A receptor stimulation and *N*-methyl-D-aspartate (NMDA) receptor antagonism, Bax translocates to mitochondria and permeabilizes the outer mitochondrial membrane (Olney et al., 2004). The combination of these events activates the mitochondrial apoptosis pathway and allows cytochrome *c* to be released into the cytosol (Fig. 1). Release of cytochrome *c* results in apoptosome formation, caspase-3 activation, DNA fragmentation, and ultimately, apoptotic neuronal cell death (Olney et al., 2004).

Low concentrations of CO can bind to the cytochrome *c*-cardiolipin complex within mitochondria and inhibit the peroxidase activity of cytochrome *c* (Fig. 1) (Kapetanaki et al., 2009). This prevents apoptosis by blocking the mobilization and release of cytochrome *c* (Kapetanaki et al., 2009; Ignarro et al., 1982). CO-mediated dose-dependent inhibition of cytochrome *c* peroxidase activity has been demonstrated in vivo in the developing brain of newborn mice following brief exposure to low concentrations of CO (Cheng et al., 2012). As detailed earlier, such postnatal low dose exposures inhibited natural programmed cell death within the murine forebrain in a concentration-dependent manner (Cheng et al., 2012). Thus, we hypothesized that the anti-apoptotic effects of sub-clinical concentrations of CO could limit anesthesia-induced neuronal apoptosis.

We tested our hypothesis in 7-day old male mice exposed to air (0 ppm CO), 5 ppm CO, or 100 ppm CO with or without isoflurane (2%) for 1 h (Cheng and Levy, 2014). The duration of exposure was chosen because 1-h exposure to 2% isoflurane had previously been shown to induce neuronal apoptosis in 7-day old mice and because it represented a relatively brief anesthetic exposure (Johnson et al., 2008). The concentrations of CO were selected based on our findings that infants and children encounter an active CO exposure during a 1-h low-flow general anesthetic; inspiring up to 20 ppm CO with a mean exposure of approximately 4 ppm (Levy et al., 2010; Nasr et al., 2010). Thus, 5 ppm CO reflected a common level of exposure during LFA, 100 ppm CO represented the upper limit of a sub-toxic exposure, while air (0 ppm CO) served as a control exposure (Cheng and Levy, 2014).

Resultant COHb levels following exposure approximated those expected in humans after similar time-weighted exposures (Cheng and Levy, 2014; Winter and Miller, 1976; Peterson and Stewart, 1970; Stewart et al., 1970). Exposure to 100 ppm CO for 1 h, for example, yielded peak COHb levels of 4.5% in both experimental cohorts (with and without isoflurane) (Cheng and Levy, 2014). Because such levels are well below values known to result in tissue hypoxia and are markedly less than levels known to elicit signs and symptoms in humans, exposure to either concentration of CO in this model represented a sub-toxic CO exposure (Kao and Nañagas, 2005; Cheng and Levy, 2014; Gorman et al., 2003).

Consistent with anesthetic-induced neuronal apoptosis, 1-h isoflurane exposure significantly increased cytochrome *c* peroxidase activity, cytochrome *c* release from mitochondria, and the number of activated caspase-3 cells and apoptotic nuclei in the newborn mouse forebrain (Cheng and Levy, 2014). In contrast, low dose CO limited these effects in a dose-dependent manner (Cheng and Levy, 2014). Specifically, CO exerted a concentration-dependent reduction in the number of activated caspase-3 positive cells in the hypothalamus/thalamus and in the number of apoptotic nuclei in the neocortex and hippocampus when administered with isoflurane (Cheng and Levy, 2014). In addition, combined exposure to CO with isoflurane significantly decreased cytosolic levels of cytochrome *c* in the forebrain in a step-wise manner compared to isoflurane exposure alone indicating dose-dependent CO-mediated inhibition of cytochrome *c* release (Cheng and Levy, 2014). Upstream from cytochrome *c* release, CO significantly decreased forebrain cytochrome *c* peroxidase activity in a concentration-dependent manner with and without isoflurane (Cheng and Levy, 2014). The findings suggest that low dose CO has the potential to limit or offset the pro-apoptotic consequences of isoflurane in the developing murine brain depending on concentration.

With regard to oxidative stress, despite evidence demonstrating that anesthetics induce ROS within mitochondria, the exact source of free radical generation has not been defined. It is known, however, that dys-regulation of COX, the rate limiting enzyme of the electron transport chain, causes complex I and III to generate superoxide radical under certain conditions (Srinivasan and Avadhani, 2012; Lee et al., 2002). For example, ROS can be produced when COX becomes overactive and hyperpolarizes the mitochondrial membrane potential as well as when COX is inhibited, resulting in electron leak from the respiratory chain (Srinivasan and Avadhani, 2012; Lee et al., 2002; Chen et al., 2003). Thus, modulation of COX activity is necessary during homeostasis to prevent oxidative stress (Cheng et al., 2015).

As a known modulator of COX, CO can inhibit the enzyme at high concentrations and stimulate COX activity at low concentrations (Cooper and Brown, 2008; Choi et al., 2012; Almeida et al., 2012). However, the role of COX and CO-mediated regulation of its activity in the context of anesthesia-induced oxidative stress in the developing brain has been relatively unexplored. Thus, in a subsequent study, we aimed to determine the effect of low concentration CO on COX specific activity in the immature murine brain during isoflurane exposure with a focus on oxidative stress (Cheng et al., 2015).

Consistent with anesthesia-induced oxidative stress, 1-h exposure to isoflurane significantly increased levels of malondialdehyde (MDA), a marker of fatty acid peroxidation, within forebrain mitochondria of 7 day old mice (Cheng et al., 2015). Interestingly, mitochondrial lipid peroxidation also increased following 1-h exposure to low concentration CO alone in both CO-exposed cohorts (5 ppm CO or 100 ppm CO) (Cheng et al., 2015). However, combined exposure to either dose of CO with isoflurane resulted in levels of mitochondrial ROS that were significantly lower than those seen following concentration-matched CO exposure alone (Cheng et al., 2015). Furthermore, MDA levels approximated control values following combined exposure to 100 ppm CO with isoflurane (Cheng et al., 2015). The findings indicated that isoflurane or low concentration CO exposure independently caused oxidative stress in the developing forebrain, while combined exposure to both agents limited mitochondrial lipid peroxidation.

With regard to enzyme activity, independent exposure to either isoflurane or low concentration CO stimulated and activated forebrain COX (Cheng et al., 2015). However, combined exposure to CO with isoflurane paradoxically limited this overactivity in a dose-dependent manner for CO (Cheng et al., 2015). Linear regression analysis between mitochondrial lipid peroxidation and COX kinetic activity demonstrated a strong and highly significant correlation, suggesting a role for COX dysregulation as an etiology for anesthesia-induced oxidative stress (Cheng et al., 2015).

COX is regulated, in part, by post-translational modification involving reversible subunit phosphorylation (Srinivasan and Avadhani, 2012). In our work, mice that underwent combined exposure to low dose CO with isoflurane demonstrated a concentration-dependent and synergistic hyperphosphorylation of tyrosine residues within COX subunit I, the active site (Cheng et al., 2015). Phosphorylation of tyrosine 304 of COX subunit I is known to strongly inhibit the enzyme (Lee et al., 2005). Because the associated directionality of change in COX activity within the forebrain of each cohort was consistent with the state of phosphorylation, the data suggested that tyrosine phosphorylation of COX subunit I might have contributed, in part, to the modulation of COX kinetic activity and oxidative stress following exposure to CO with or without isoflurane (Cheng et al., 2015). Thus, low concentration CO may prevent anesthesia-induced neuronal apoptosis by exerting antioxidant effects within mitochondria as well as by inhibiting the peroxidase activity of cytochrome *c* (Fig. 1).

Importantly, the findings provide some insight into the dual effects of CO as a potential neurotoxin versus neuroprotectant. The cellular consequences of CO depend on the context, duration, concentration, and timing of exposure. However, this relationship is not unique to CO. A number of other biologically active agents have been shown to exert similar properties; inducing injury in certain settings, while paradoxically conferring neuroprotection in other contexts. This dichotomy has been observed with lithium, cannabinoids, NMDA receptor activation, and anesthetics, themselves (Wei et al., 2007; Fountoulakis et al., 2008; Papadia and Hardingham, 2007; Bologov et al., 2011). The balance between cytoprotection and cell death is thought to be related to the effects on oxidative stress and calcium signaling (Papadia and Hardingham, 2007). Our findings

regarding mitochondrial lipid peroxidation following exposure to low concentration CO alone or in combination with isoflurane are consistent with this notion.

6. Conclusions

Anesthesia-induced neurotoxicity may result from activation of the oxidative stress-associated mitochondrial apoptosis pathway (Olney et al., 2004; Yon et al., 2005; Bai et al., 2013; Boscolo et al., 2013; Zhang et al., 2010). Low dose CO targets the peroxidase activity of cytochrome *c* and modulates COX kinetics in the developing brain during anesthesia exposure. Depending on CO concentration, combined exposure with a volatile agent can prevent anesthesia-mediated oxidative stress in fore-brain mitochondria and inhibit cytochrome *c* release, thereby limiting free radical generation and blocking activation of the intrinsic apoptosis pathway. CO-mediated inhibition of these upstream events is ideal given that release of cytochrome *c* represents the “point-of-no-return” in the pathway (Ferraro et al., 2008).

Because infants and children are commonly exposed to low levels of CO during LFA, development of CO as a potential preventative therapy for anesthesia-induced neurotoxicity is clinically relevant and such exposures could be seamlessly incorporated into routine anesthetic management. However, taken as a whole, the preclinical data indicate that low concentrations of CO are biologically active in the developing brain and have the potential to provide either neuroprotection or cause neurotoxicity depending on the context, duration, and concentration of exposure. It is important to note that animal studies have demonstrated that exposure to low concentration CO, as a sole agent, disrupts vital neurodevelopmental processes and causes oxidative stress in young mammals. Because the immature brain is vulnerable during the critical window of development, further investigation of the safety and efficacy of such CO exposures is necessary. Future work will, therefore, need to focus on the neurodevelopmental consequences of low concentration CO exposure in the setting of an anesthetic and need to explore outcomes following exposures that commonly occur during LFA in humans. Ultimately, however, as we understand more about the toxicity of anesthetics and the biological activity of CO in the developing brain, low dose CO may emerge as therapeutic tool in our armamentarium of gases that will protect the infants and children we care for.

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Fig. 1.

Oxidative stress-associated mitochondrial apoptosis pathway (adapted from ref. Levy (2015)). A model of mitochondrial derived-ROS linked to the intrinsic apoptosis pathway is depicted. ROS are generated within mitochondria during oxidative phosphorylation. The majority of superoxide radicals (O_2^-) are produced by complexes I and III of the electron transport chain during aerobic respiration when electrons are transferred to molecular oxygen. Dysregulation of cytochrome oxidase (COX) enhances such ROS production. Following their formation, superoxide radicals are converted to hydrogen peroxide (H_2O_2) in the mitochondrial matrix by manganese-dependent superoxide dismutase (MnSOD) or by copper-zinc superoxide dismutase (CuZnSOD) in the intermembrane space. In the presence of H_2O_2 , cytochrome *c* (c) oxidizes cardiolipin (CL) to hydroperoxycardiolipin (CL-OOH). This permits cytochrome *c* to become unbound from CL and to mobilize from the inner mitochondrial membrane. Following Bax permeabilization of the outer mitochondrial membrane, cytochrome *c* is released into the cytosol, which, ultimately activates caspase-3. Anesthetic exposure activates COX, induces mitochondrial oxidative stress, increases cytochrome *c* peroxidase activity, and results in Bax translocation to mitochondria. This combination of events allows cytochrome *c* to be released into the cytosol to form the apoptosome and activate caspase-3. Carbon monoxide (CO) modulates anesthesia-mediate COX activation, limits mitochondrial ROS formation, and binds to the cytochrome *c*-cardiolipin complex to inhibit anesthesia-induced peroxidase activity of cytochrome *c*. These effects limit activation of the mitochondrial pathway of apoptosis and prevent release of cytochrome *c* into the cytosol in a concentration-dependent manner for CO.

Table 1

Types of carbon monoxide (CO) exposure (Winter and Miller, 1976; Raub and Benignus, 2002; Tomaszewski, 2002). Resultant carboxyhemoglobin (COHb) levels depend on the concentration and the duration of CO exposure. Sub-toxic exposure refers to threshold required to induce signs of tissue hypoxia.

Type of CO exposure	CO Concentration (ppm)	Resultant COHb level (%)	Signs and symptoms
Low concentration, sub-toxic, sub-clinical	10	2	Asymptomatic
	70–120 for ~4 h	10–20	Relatively asymptomatic, without signs of tissue hypoxia
Intermediate	200–800	~30–50	Headache, dizziness, and impaired judgment
High concentration, toxic	>800	>60	Seizures, coma, and death

CO, carbon monoxide; ppm, parts per million; COHb, carboxyhemoglobin.

Table 2

Biological activity of low dose carbon monoxide (CO) in various organ systems and cell types.

CO exposure	Organ system/cell type	Model	Effect(s)	Reference(s)
250 ppm	Lungs	Rat model of hyperoxia-induced lung injury	Attenuated neutrophil accumulation and apoptosis	Otterbein et al., 1999
Up to 250 ppm	Lungs	Ventilator-induced lung injury	Reduced pro-inflammatory cytokines and total cell number in BAL fluid while increasing levels of the anti-inflammatory cytokine, interleukin (IL)-10	Dolinay et al., 2004
100–300 ppm	Lungs	Baboon pneumococcal pneumonia	Accelerated the resolution of acute lung injury	Fredenburgh et al., 2015
500 ppm for 6 h	Lungs	Mouse model of acid aspiration	Significantly reduced the number of neutrophils in the BAL fluid and decreased the magnitude of lung injury	Nemzek et al., 2008
100–125 ppm for 2 h a day for 4 consecutive days	Lungs	Human patients with chronic obstructive pulmonary disease	Trend toward reduced eosinophils within the sputum and enhanced airway responsiveness	Bathoorn et al., 2007
Low dose CO (various concentrations)	Lungs	Various models of lungs ischemia–reperfusion	Blunted the inflammatory response and prevented apoptosis	Fujita et al., 2001; Zhang et al., 2005; Kohmoto et al., 2007; Mishra et al., 2006; Song et al., 2003; Zhang et al., 2003
Low dose CO (various concentrations)	Lungs	Various animal models of lung transplantation, CPB, and secondary lung injury	Pro-survival effects	Kohmoto et al., 2007; Song et al., 2003; Boutros et al., 2007; Goebel et al., 2008
Pre-occlusion exposure to low dose CO	Heart	Rat model of coronary artery occlusion	Reduced infarct size, suppressed inflammatory cell migration into the area at risk, and decreased the expression of pro-inflammatory cytokines	Fujimoto et al., 2004
250 ppm CO for 2 h prior to cardiac arrest along with administration of CO-saturated cardioplegia solution	Heart	Porcine CPB model	Enhanced myocardial bioenergetics, reduced interstitial edema and cardiomyocyte apoptosis, and led to fewer number of electrical cardioversions required following reperfusion	Lavitrano et al., 2004
Exposed the donor and the recipient to 250–400 ppm	Heart	Model of mouse-to-rat cardiac transplantation	Reduced graft rejection and promoted long-term graft survival	Sato et al., 2001
Expose donors to 400 ppm prior to transplantation and subjected the explanted heart to storage solution saturated with 1000 ppm	Heart	Rat cardiac transplantation	Protected the graft from ischemia-reperfusion injury via an anti-apoptotic mechanism	Akamatsu et al., 2004

CO exposure	Organ system/cell type	Model	Effect(s)	Reference(s)
Exposed recipients to 20 ppm CO post-transplant	Heart	Rat heterotopic heart transplant	Significantly prolonged allograft survival	Nakao et al., 2006
Preconditioned with 250 ppm	Cultured mouse neurons	Apoptotic-induced death	Enhanced survival and cellular protection	Vieira et al., 2008
250 ppm for 3 h, 1 day prior to CPB with deep hypothermic circulatory arrest	Brain	Piglets CPB with deep hypothermic circulatory arrest	Completely prevented cell death in the neocortex and hippocampus	Mahan et al., 2012
Preconditioned with 250 ppm	Brain	Newborn mouse model of hypoxia and ischemia	Protected cells within the hippocampus from hypoxia- and ischemia-induced apoptosis	Queiroga et al., 2012
Post-conditioned with 250 ppm for 18 h	Brain	Complete occlusion of the middle cerebral artery in mice	Reduced infarct size by about 30%	Wang et al., 2011

ppm, parts per million; CO, carbon monoxide; BAL, bronchoalveolar lavage; CPB, cardiopulmonary bypass.