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BRAIN RESEARCH

Research Report

Hypoxia-independent apoptosis in neural cells exposed to carbon monoxide *in vitro*

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ABSTRACT

The neurotoxic effects of carbon monoxide (CO) are well known. Brain hypoxia due to the binding of CO to hemoglobin is a recognized cause of CO neurotoxicity, while the direct effect of CO on intracellular targets remains poorly understood. In the present study, we have investigated the pathways leading to neural cell death induced by *in vitro* exposure to CO using a gas exposure chamber that we have developed. Mouse hippocampal neurons (HT22) and human glial cells (D384) were exposed to concentrations of CO ranging from 300 to 1000 ppm in the presence of 20% oxygen. Cytotoxicity was observed after 48 h exposure to 1000 ppm, corresponding to approximately 1 μM CO in the cultured medium, as measured by gas chromatography. CO induced cell death with characteristic features of apoptosis. Exposed cells exhibited loss of mitochondrial membrane potential, release of cytochrome c into the cytosol, nuclei with chromatin condensation, and exposure of phosphatidyl serine on the external leaflet of the plasma membrane. CO also triggered activation of caspase and calpain proteases. Pre-incubation with either the pancaspase inhibitor Z-VAD-fmk (20μM) or the calpain inhibitor E64d (25 μM) reduced by 50% the occurrence of apoptosis. When pre-incubating the cells with the two inhibitors together there was an additional reduction in the number of cells with apoptotic nuclei. These data suggest that CO causes apoptosis via activation of parallel proteolytic pathways involving both caspases and calpains. Furthermore, pre-treatment with the antioxidant MnTBAP (100 μM) significantly reduced the number of apoptotic nuclei, pointing to a critical role of oxidative stress in CO toxicity.

1. Introduction

Carbon monoxide (CO) is an endogenously produced gas generated by oxidation of organic molecules and degradation of heme. It has important physiological roles in modulation of certain neuronal processes, including intercellular signal transduction (Verma et al., 1993) and generation of long-term potentiation (LTP) in hippocampus (Alkadhi et al., 2001; Zhuo et al., 1993). However, poisoning from exposure to exogenous CO such as accidental poisoning from home heating, automobile exhausts and smoke, as well as intentional for suicidal purposes occurs frequently. Once CO enters the bloodstream through the lungs, it attaches to hemoglobin (Hb), forming carboxyhemoglobin (COHb) and thereby reduc-

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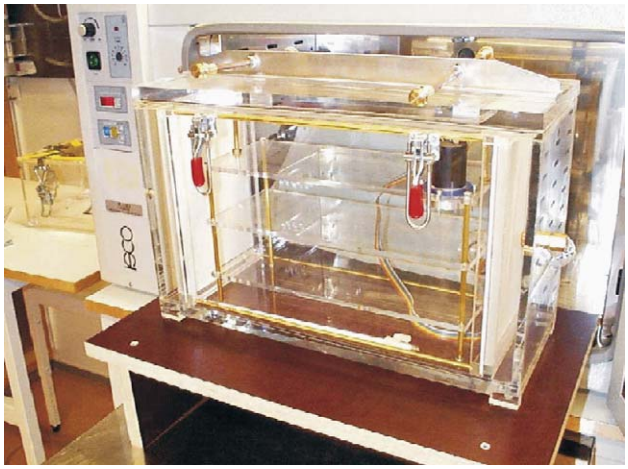

Fig. 1 – Front view of the test chamber.

ing oxygen (O_2) transport with subsequent hypoxia. The acute symptoms of CO poisoning depend on severity of exposure and include headache, dizziness, nausea, vomiting, confusion, impaired language and cognition, collapse, and coma. At lower concentrations, CO affects visual perception, manual deftness, learning and attention level (see review Raub and Benignus, 2002; Gorman et al., 2003).

Perhaps, the most insidious effects of CO are on the developing central nervous system. Children of women smoking during pregnancy have lowered intellectual development (Frydman, 1996), which could be a consequence of exposure to CO via smoke. Experimental studies have shown that prenatal exposure to low concentrations of CO lead to disrupted hippocampal long-term potentiation (LTP) (Alkadhi et al., 2001; Zhuo et al., 1993; Mereu et al., 2000), and altered habituation and working memory (Giustino et al., 1999; Mactutus and Fechter, 1984).

Brain hypoxia has been considered the major cause of CO neurotoxicity, but in addition, CO can exert a direct damage to cells, possibly by binding directly to intracellular targets such as cytochrome P450 mono-oxygenase and cytochrome *c* oxidase (Uemura et al., 2001). However, it is still unclear to what extent cell death induced by CO may be due to a direct effect not involving hypoxia.

In an attempt to characterize the mechanism(s) of CO toxicity, we have used an *in vitro* exposure system to investigate the intracellular pathways leading to neural cell death induced by CO. The mouse hippocampal cell line HT22 and the human astrocytoma cell line D384 were exposed to different CO concentrations under normoxic conditions using an exposure chamber that we have developed to expose cultured cells to volatile agents (Fig. 1).

2. Results

2.1. *Cell damage induced by CO*

We focused our studies on the neuronal HT22 and glial D384 cell lines because hippocampal and glial cells are known CO targets *in vivo* (Piantadosi et al., 1997). To investigate the occurrence of cell death, HT22 and D384 cells were exposed to different concentrations of CO ranging from 300 to 1000 ppm for 24 to 48 h in both cell lines. Cytotoxic effects such as abnormal cell attachment and cell shrinkage were detected after exposure to 1000 ppm for 48 h (Figs. 2A, E and B, F). Nuclear staining of fixed cells showed characteristic alterations, i.e., condensed chromatin and intensively propidium iodiode (PI) stained nuclei (Figs. 2C, G and D, H), hallmarks of apoptosis (Kerr et al., 1972). Additional tests with Trypan blue showed that CO induced a significant decrease in the total cell number only after exposure to 1000 ppm for 48 h (Fig. 2I) with no alterations of cell membrane permeability (Fig. 2J), confirming that apoptosis and not necrosis was the type of cell death occurring in the exposed cells.

2.2. *Phosphatidylserine (PS) exposure on the plasma membrane*

Translocation of PS to the outer leaflet of the plasma membrane plays a critical role in phagocytosis of apoptotic cells (Fadok et al., 1992). To characterize the effects of CO on PS exposure, HT22 and D384 cells were triple stained with Hoechst 33358, Annexin V and PI. Exposed HT22 and D384 cells exhibited nuclear condensation with PS translocation, which were visualized with the vital dye Hoechst 33358 and Annexin V (Figs. 3D, E and J, K). As expected, cells were not stained by the cell-impermeant dye PI, indicating that the plasma membrane was intact and cells were indeed undergoing apoptosis (Figs. 3F, L).

2.3. *Loss of mitochondrial membrane potential and release of cytochrome c*

We used the vital dye tetramethylrhodamine ethyl ester (TMRE) to evaluate the mitochondrial potential. HT22 cells and D384 cells exposed to CO for 48 h showed loss of membrane potential, resulting in the lack of mitochondrial TMRE staining in cells with condensed nuclei (Figs. 4A–B and E–F). To determine further alterations in mitochondrial function, we used immunocytochemistry to look at cytochrome *c* (cyt *c*) release into cytosol. The release of this protein from mitochondria to the cytosol plays an essential role in the formation of the apoptosome complex with subsequent activation of the caspase cascade executing apoptosis (Li et al., 1997). In control HT22 and D384 cells cyt *c* was localized in the mitochondria, whereas in CO exposed cells mostly in the cytosol (Figs. 4C–D and G–H).

2.4. *Activation of caspases and calpains*

Caspases, a family of cystein proteases cleaving after aspartate residues, play an important function in neuronal apoptosis (Gorman et al., 1998). Therefore, we investigated the role of caspases in CO-induced apoptosis by measuring their proteolytic activity. Exposure to CO induced a significant increase in caspase 3-like activity in whole cell extracts from both cell lines, approximately 5 folds in HT22, and 9 folds in D384 cells (Fig. 5A). Calpains are cysteine proteases activated by changes in intracellular calcium concentrations. Calpains can either inactivate caspases or participate together with caspases in