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Xanthine oxidoreductase and neurological sequelae of carbon monoxide poisoning

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Introduction

Carbon monoxide is the leading cause of injury and death by poisoning worldwide (Kao and Nanagas, 2005). The clinical symptoms of CO poisoning are often nonspecific and diverse, and not confined to immediate responses. About 13–50% of severe CO poisoning patients, with loss of consciousness or carboxyhemoglobin level higher than 25%, will develop neurological sequelae after a latency period of 2 days to 6 weeks (Hsiao et al, 2004; Thom et al, 1995; Weaver et al, 2002). Neurological sequelae (NS) commonly encountered include difficulties with higher intellectual functions, impaired short-term memory, gait disturbance, seizures, cortical blindness, emotional lability, hallucinations, and psychosis (Kao and Nanagas, 2005).

Our past reports have shown that immune events induced by alterations in myelin basic protein (MBP) lead to NS (Thom et al, 2004a), and platelet-neutrophil interaction precipitates the changes in MBP (Thom et al, 2006). In the cascade of events leading to MBP modifications, lipid peroxidation plays a key role. Conversion of xanthine dehydrogenase (XDH) to xanthine oxidase (XO) has been reported to be an important component for generating reactive species required for lipid peroxidation (Thom, 1992).

XDH and XO are molybdenum-containing enzymes collectively referred to as xanthine oxidoreductase (XOR). XOR is widely expressed in mammalian tissues (Wajner and Harkness, 1989), and is largely localized to the vascular endothelium of brain (Betz, 1985). XOR is involved in purine catabolism and in conditions such as ischemia-reperfusion injury or cardiovascular disease may generate reactive oxygen species (Granger et al, 1986, Berry and Hare, 2004). XDH is the major form existing in mammalian cells and is converted to XO either by oxidation of cysteine residues or proteolysis (Nishino et al, 2005). Previous studies have shown that XO increases after CO poisoning and generates reactive oxygen species that cause lipid peroxidation (Thom, 1992).

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Although data have suggested that XOR plays a role in initiation of lipid peroxidation, there is no direct evidence linking XOR with MBP alterations and CO-mediated neurological sequelae. To address this gap, we examined the effect of CO poisoning in a group of rats fed a tungsten supplemented molybdenum-free diet. The result of feeding a diet containing tungsten is incorporation of tungsten instead of molybdenum into newly synthesized XDH and XO, resulting in progressive loss of oxidase activity. Complete inactivation can be achieved when tungsten is fed for more than 14 days (Johnson et al, 1974, and Topham et al, 1986).

Materials and methods

Animal manipulations

Wistar male rats (Charles River Laboratories, Wilmington, MA) weighing 190–300 g were divided into 6 treatment groups. Control animals fed a standard diet and water ad libitum breathed air (Control-air) or CO (Control-CO). Sham animals fed a protein diet and deionized water breathed air (Sham-air) or CO (Sham-CO). Treatment animals fed a protein diet supplemented with 0.7g/kg sodium tungstate (ICN Biochemicals, Cleveland, OH) and deionized water with 0.6 mM sodium tungstate (XOR-depleted group) for 30 days breathed air (XOR-depleted-air) or CO (XOR-depleted-CO). Rats fed the tungsten-supplemented diet grew at the same rate as the other groups.

Exposure to CO in this study followed our previously described model (Thom, 1992). Rats were placed in a 7-liter Plexiglas chamber, into which 60ml of pure CO was injected to pre-balance the chamber. A mixture of 1,000 ppm CO in air (Air Products, Allentown, PA) was flushed through at a rate of 8–12 l/min for 40 min, at which time the gas was switched to 3,000 ppm CO in air and another 60ml CO bolus was added. The majority of rats lost consciousness in this environment within 20 min and any rats that did not fall unconscious by 20 min were rendered unconscious with a 60ml bolus of pure CO. Rats usually regained consciousness in 4 min after being moved to room air.

Tissue preparation for column chromatography

Rats were anesthetized and brain tissues were processed for column chromatography as described previously (Thom et al, 2004a). In brief, isolated brain was frozen in liquid nitrogen, homogenized in 5 ml chloroform:methanol (2:1 v/v), and then diluted with chloroform:methanol to obtain a ratio of 1 g brain: 19 ml ice-cold chloroform:methanol. This volume of solvent was defined as “one volume” for subsequent steps. Following centrifugation (2000 x g for 5 min) and washing twice in one volume of fresh chloroform:methanol, the residue was washed once in ice-cold acetone. Following centrifugation (2000 x g for 5 min), the pellet was washed three times with 10 ml water, and then suspended in 10 volumes of 0.03 N HCl. The suspension was incubated for 1 h at 4 °C while stirring, centrifuged at 44,000 x g, and material in the clear aqueous-acid extract obtained by overnight vacuum evaporation. The lyophilized sample was dissolved in 500 µl 0.1 M acetic acid and applied to a carboxymethylcellulose column equilibrated with 0.1 M acetic acid. Protein was eluted using a linear gradient of sodium chloride (0–0.75 M) in 0.1 M acetic acid and collecting as 300 µl fractions. Protein concentration in the fractions was determined by absorbance at 280 nm.

Maze studies

Rat cognitive function was examined using an 8-arm wooden radial maze as previously described (Thom et al, 2004b), following procedures first described by Levin (Levin et al, 1993). In brief, familiarization and testing procedures were performed every other day, 3 days per week. CO exposure occurred after the third familiarization session on Friday afternoon and rats were left undisturbed for 2 days. Testing started the following Monday and was performed for 4 weeks (12 test sessions) by a tester blinded to which groups rats were classed. Rats were

deprived of rat chow for 23 h prior to each test and each maze arm was baited with food reinforcement (a Kellogg's Froot Loop) located 2 cm from the end. Rats were held by the tester for 5 min, fed 1/2 of a Froot Loop, and then placed in the maze central octagon. After 10 s, a stopwatch was started and rats maneuvered down maze arms to eat the Froot Loop. An arm choice was recorded when all four paws were placed into an arm. If a rat reentered an arm, it was recorded as an error. The testing session continued until the rat entered all 8 arms or 5 min had elapsed. Data recorded were number of entries until a repeat occurred (choice accuracy), reentries, and the time to complete the session (in seconds) divided by the total number of arm entries (latency or response duration). The maze was wiped with 1% acetic acid solution before each session to remove scents left by a previously tested rat.

Statistics

Statistical analysis of maze scores was performed by repeated measures ANOVA followed by Tukey test (SPSS 11, SPSS Inc, Chicago, IL). The level of significance was taken as $P < 0.05$.

Results

Column chromatography

Cation exchange chromatography resolved the typical six peaks elution pattern of MBP for acid soluble material obtained from Control-air rats (Fig. 1). Material obtained from Control-CO rats demonstrated loss of most positively charged peaks. The MBP charge pattern of each Sham group was similar to that of corresponding Control group. In contrast, the MBP charge patterns of XOR-depleted groups were virtually the same whether rats breathed air or CO.

Radial maze

Improvement of maneuvering in an eight-arm baited radial maze was assessed as the 'entry-to-repeat' score and significant improvements were found in Control-air group, XOR-depleted-air group, and XOR-depleted-CO group ($p < 0.05$, Fig. 2a). In Control-CO and Sham-CO groups, 'entry-to-repeat' scores failed to improve over the four week testing period and were significantly different from the score of XOR-depleted-CO rats ($p = 0.003, 0.036$). Latency scores were highly variable in all groups, and no statistically significant difference was noted among five groups (Fig. 2b).

Discussion

Results from this investigation indicate that XOR plays an essential role in neurological dysfunction following CO poisoning. CO poisoned XOR-depleted rats did not exhibit changes in MBP charge pattern that are linked to immune mediated neuropathology. Consistent with this finding, XOR-depleted rats did not exhibit cognitive impairment after CO poisoning.

In this study, the XOR-depleted-CO rats showed gradual improvement in 'entry-to-repeat' scores, which suggests that after CO exposure they had learning abilities that were similar to non-poisoned rats. On the contrary, Control-CO and Sham-CO rats showed no improvements in 'entry-to-repeat' scores over the four week testing period. Although maze studies do not test all the basic systems that influence cognition, these results demonstrate the linkage between XOR and CO-mediated neurological dysfunction.

The disparity of cognitive function among five groups in this investigation is not significant until the middle of the 4th week, i.e. day 24. This suggests that the timing for developing NS in the rat model is close to clinical observations with CO poisoning in patients, who develop NS after a lucid interval of 22.5 days on average (Min, 1986). The incidence of NS in patients

increases with the duration of unconsciousness during acute CO intoxication and about 90% DNS patients have suffered a period of unconsciousness (Choi, 1983).

The results of this study indicate the necessity of XOR activity in pathophysiology of CO-mediated NS. Taken in conjunction with previous trials, the data indicate that intravascular platelet-neutrophil interactions and subsequent neutrophil activation with attachment to the vascular walls are the initial events occurring during and immediately following CO poisoning. We previously showed that neutrophil activation/adherence to the vascular wall was required for XDH conversion to XO, and that XO activity was required for brain lipid peroxidation. Aldehydic lipid peroxidation products, such as malonaldehyde react with amino acid residues of MBP, alter the MBP structure and precipitate immunological responses leading to neurological sequelae.

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Figure 1.

Column chromatography analysis.

Panels show the elution pattern of brain acid-soluble material obtained from rats of Control-air group, Control-CO group, Sham-air group, Sham-CO group, XOR-depleted-air group, and XOR-depleted-CO group. High fraction numbers indicate more positively charged proteins, totally 200 fractions in each sample. ABS=absorbance. Open squares are mean $\pm$ SE; n=4 in Control-air and Control-CO groups, n=3 in others.

Figure 2.

Maze test scores.

(a) Entry-to-repeat scores, asterisk symbolizes scores significantly increasing after four week test period ($p < 0.05$). (b) Latency scores, no statistical difference among five groups.

(SE=standard error. Sample number: Control-air group =10, Control-CO group =10, XOR-depleted-air group =4, XOR-depleted-CO =10, Sham-CO =4)