Protective effect of erythropoietin on myocardial apoptosis in rats exposed to carbon monoxide

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A R T I C L E   I N F O

Article history:
Received 4 October 2015
Received in revised form 2 February 2016
Accepted 3 February 2016
Available online 23 February 2016

Keywords:
Carbon monoxide
Erythropoietin
Myocardial apoptosis
Bcl2

A B S T R A C T

Aims: Cardiac complications are common in carbon monoxide (CO) poisoning and associated with high morbidity and mortality. We have previously shown that erythropoietin (EPO) could reduce CO-induced cardiac ischemia in rat. In the current study, the anti-apoptotic effect of EPO during CO cardiotoxicity was investigated in order to elucidate the mechanism of EPO anti-ischemic action.

Main methods: Wistar rats were exposed to CO (250, 1000 and 3000 ppm). EPO (5000 IU/kg) was administered to all groups by intraperitoneal injection at the end of CO exposure period. TUNEL and caspase-3 activity levels were assessed to investigate the effects of CO exposure and subsequent EPO administration on myocardial apoptosis. The changes of mitochondrial membrane potential (MMP) were also assessed with sensitive lipophilic dye JC-1 by flow cytometry. The roles of Bcl2 and Bax in EPO protective effect were investigated by Western blotting.

Key findings: Myocardial apoptosis was observed following CO exposure. Moreover, mitochondrial membrane depolarization and significant reduction in Bcl2/Bax ratio were shown following CO poisoning especially at 3000 ppm. On the other hand, EPO administration could effectively suppress apoptosis in myocardial cells. Also, EPO significantly prevented the CO-induced depolarization of MMP (p < 0.001) and preserved Bcl2/Bax ratio (p < 0.01).

Significance: EPO reduces myocardial injury due to CO intoxication. Thus EPO could be suggested as a possible candidate for the management of CO cardiotoxicity with clinical applications.

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1. Introduction

Carbon monoxide (CO), although necessary for cell signaling, could lead to significant health problems after exposure to high concentrations or even chronic low exposure. CO sources vary from second hand smoking to vehicular exhaust, industrial emissions, poorly ventilated gas heaters contributing to air pollution [1]. Acute CO poisoning in relative high concentrations is a potentially life-threatening condition. Cardiac and nervous tissues are mainly affected from CO. Cardiotoxicity and myocardial injury occur due to moderate and severe CO exposure [2, 3], with the most common manifestations being tachycardia, ischemia, dysrhythmia, infarction and cardiac arrest in severe cases [4-6]. Ischemic electrocardiographic (ECG) changes have been reported following CO poisoning even in patients with few cardiac risk factors [7, 8]. The long term mortality from myocardial disorders in CO poisoning is twice that of patients with no cardiac involvement and three times that of negative controls [3]. Recently myocardium involvement in CO exposure has been identified as CO related cardiomyopathy [7]. A link between ambient CO air pollution and heart failure in the elderly has been found. Burnett et al. reported that the rise in ambient air CO levels correlated with exacerbations in hospital admission for heart failure [8].

While CO heart effects range from mild to myocardial fibrosis and contractile dysfunction, with the main pathophysiologic process considered being hypoxic damage, a number of cardiосpecific mechanisms at cellular or subcellular level are implicated to myocardial injury. CO
acts directly to myocardial mitochondria through impairment of mito-
chondria respiratory chain at the cytochrome C oxidase level [4–5]. In
cultures of endothelial cells exposed to increasing CO concentrations,
apoptosis resulted with concurrent caspase-1 rise. On the other hand,
addition of a caspase-1 inhibitor reduced endothelial cell death [9]. On
a tissue level, apoptotic cell death due to CO intoxication has been
described in nervous tissues [10], however there are limited data
concerning CO-induced cardiac apoptosis.

Recently, erythropoietin (EPO), a hematopoietic cytokine, has been
investigated extensively as an anti-ischemic and tissue-protective
agent [11, 12] with cardioprotective action [13–16] that is unrelated to
its erythropoietic property [14]. In cardiac ischemia/reperfusion studies,
EPO administration improved ventricular and cardiac hemodynamic
functions and led to reduction of infarct size, inhibition of myocardial
apoptosis and normalization of hypertrophic index [14, 17]. In vivo
and in vitro studies have shown that the anti-apoptotic mechanism
has a main role in EPO tissue protective effect [15, 18].

2. Methods

2.1. Animal

Wistar male rats, weighing 200–250 g were housed in the Animal
Center of BuAli Research Institute. They were kept under standard con-
ditions (21–23 °C temperature, 12 h/12 h light/dark cycle) with free ac-
dess to food and water. All animals were treated in accordance with the
Guidelines for the Care and Use of Laboratory Animals prepared by the
National Academy of Sciences and published by the National Institutes
of Health (NIH publication no. 85-23, Revised 1996) and in conformity
with EU Directive 2010/63/EU for animal experiments. The study was
conducted in accordance with the EU directive 2010/63/EU for animal
experiments and the animal care regulations of the relevant animal
group. All animals were treated in accordance with the relevant Directive
21°C temperature, 12 h/12 h light/dark cycle) with free ac-

2.2. Experimental groups and study design

The animals were intoxicated by three different CO concentrations;
250 ppm, 1000 ppm and 3000 ppm. These three different concentra-
tions and times of CO exposure were chosen on the basis of previous
studies to induce severe (3000 ppm/40 min), moderate (1000 ppm
CO/40 min) and mild (250 ppm/90 min) CO poisoning in rats, respective-
ly [20–22]. The animals were placed in a 12L airtight plexiglass
container with entrance and exit taps. CO was flowed to the container at
a constant flow for different times based on the relevant animal group
(Table 1). The CO concentration was monitored continuously with a
CO analyzer (model 707 carbon monoxide analyzer, TPI, Korea). At the
end of CO exposure, recombinant human EPO (rEPO, Pooyesh Daraou
Co, Iran; 5000 IU/kg) was injected intraperitoneally [18]. EPO dosing
scheme was decided based on previous published studies [13, 14, 18].
In all groups, the animals were exposed to ambient air for 2 h after CO
exposure. The animals’ heart was removed after anesthesia induction
by Ketamin/Xylazin mixture (100/10 mg/kg). Animal groups are de-
scribed in Table 1.

2.3. Toxicological analysis

Blood samples were taken from the abdominal aorta of animals and
heparinized. Carboxyhemoglobin levels were assessed with a spectrop-
hotometer calibrated for rat blood (Jasco, Japan).

2.4. Determination of caspase-3 activity in the heart

Activity of caspase-3 in the heart was measured by using a caspase-
3/CDP3 fluorometric assay kit (Biovision, USA). In brief, the heart tis-

2.5. In situ detection of apoptosis (TUNEL)

To determine myocardial apoptosis, in situ cell death detection kit
(Roche, Germany) was used for the TdT-mediated dUTP-biotin nick
end labeling (TUNEL) test. Hearts were removed after reoxygenation,
flushed with cold PBS and fixed in formaldehyde 10%. Fixed tissues were
digested with proteinase K (20 μg/ml) and then washed with PBS. The slides were then incubated with terminal deoxynucleotidyl transferase
(TdT) reaction mixture for 1 h at 37 °C in a humidified chamber. The nonspecific bindings were avoided by incubation with BSA 3% in PBS for 20 min. Then, the slides were incubated with anti-fluorescein antibody conjugated with peroxidase for 30 min
at 37 °C. By adding diamobenzidine solution as a substrate, the apo-
ptotic cells were visualized as brown-colored cells. The slides were
counterstained with hematoxylin, mounted and viewed in a light mi-
croscope (Olympus, Japan). Apoptosis index was assessed from 50

Table 1

<table>
<thead>
<tr>
<th>Group</th>
<th>CO exposure time-schedule</th>
<th>EPO administration</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>3000 ppm CO</td>
<td>NO</td>
</tr>
<tr>
<td>2</td>
<td>1000 ppm CO</td>
<td>NO</td>
</tr>
<tr>
<td>3</td>
<td>250 ppm CO</td>
<td>NO</td>
</tr>
<tr>
<td>4</td>
<td>3000 ppm CO + EPO</td>
<td>5000 IU/kg (immediately after intoxication)</td>
</tr>
<tr>
<td>5</td>
<td>1000 ppm CO + EPO</td>
<td>5000 IU/kg (immediately after intoxication)</td>
</tr>
<tr>
<td>6</td>
<td>250 ppm CO + EPO</td>
<td>5000 IU/kg (immediately after intoxication)</td>
</tr>
<tr>
<td>7</td>
<td>EPO</td>
<td>5000 IU/kg</td>
</tr>
<tr>
<td>8</td>
<td>Control</td>
<td>NO</td>
</tr>
</tbody>
</table>
randomly microscopic fields for each case. Assay was performed in a blind manner.

2.6. Mitochondria isolation

Mitochondria were isolated from fresh rat’s heart using a commercial mitochondrial isolation kit (Sigma-Aldrich Chemie, USA). Briefly, the fresh rat heart was washed twice by two volumes of extraction buffer (10 mM HEPES, 200 mM mannitol, 70 mM sucrose, and 1 mM EGTA, pH 7.5). The heart was cut into small portions and suspended with 10 volumes of extraction buffer containing 0.25 mg/ml trypsin, incubated on ice for 3 min and spun. The pellet was resuspended in extraction buffer containing trypsin and incubated for 20 min on ice, added 10 mg/ml albumin, then spun again and after washing by extraction buffer, the sample was homogenized using a pestle and glass tube, then the homogenate was centrifuged at 600g for 5 min at 4°C. The supernatant was collected and centrifuged at 11,000g for 10 min at 4°C. Isolated mitochondria were pelleted and resuspended in respiratory buffer (10 mM HEPES, pH 7.4, 250 mM sucrose, 1 mM ATP, 0.08 ADP, 5 mM sodium succinate, 2 mM K2HPO4, 1 mM DTT). Mitochondrial protein was estimated by the BCA method in accordance with the manufacturer’s instructions.

2.7. Detection of mitochondrial membrane potential (MMP) (\(\Delta \Psi_m\))

MMP was evaluated by measuring the fluorescence change of 5, 5′, 6, 6′-tetrachloro-1, 1′, 3′, 3′-tetraethylbenzimidazol-carboxycyanine iodide (JC-1) in a flow cytometer. JC-1, a lipophilic fluorescent cation, sensi-
tively incorporates into the mitochondrial membrane where it can form aggregates due to the physiological membrane potential of mitochon-
dria. JC-1 aggregation leads to a shift from green to orange fluores-
cence. Isolated mitochondria were incubated with JC-1 (0.2 µg/ml) at room temperature in dark for 7 min. Changes in JC-1 signals were ana-
lyzed in a flow cytometer (FACSCalibur, Becton Dickinson, USA). For each experiment data from 20,000 mitochondria were recorded (gated on FSC-SSC parameter). Fluorescent changes were calculated at FL-1 for JC-1. Total depolarization of the \(\Delta \Psi_m\) by valinomycin (0.5 µg/ml) was used as positive control.

2.8. Western blot

Heart tissues were lysed at 4°C in 50 mM Tris buffer containing 2 mM EDTA, 2 mM EGTA, 10 mM NaF, 1 mM sodium orto-vanadate, 10 mM glycerophosphate, 10 mM 2-ME, sodium deoxycholate, PMSF and protease inhibitor. The lysate was centrifuged at 10,000g at 4°C and the supernatant was used as cellular proteins. Protein content of su-
pernatant was determined by Bradford protein assay (thermo, USA). Subsequently, an equal protein amount of each sample (50 µg) was sepa-
rated on a 12% SDS-PAGE and blotted to PVDF membrane. The non-
specific protein binding was avoided by treating the membranes by 5% skim milk in Tris-buffered saline containing 0.1% Tween-20 for 3 h at room temperature. Membranes were incubated with monoclonal anti-
odies for anti-Bcl2 (1:1000; Abcam, USA) and anti-Bax (1:1000; Abcam, USA) for 3 h at room temperature, washed three times with PBS buffer containing 0.1% Tween-20 and then incubated with the sec-
ondary antibody conjugated to horseradish peroxidase (anti-mouse IgG antibody, 1:20,000; Abcam, USA) and detected with an enhanced chemiluminescence system (ECL, Thermo, USA). The density of each protein band was determined using a gel-doc system (Alliance, Germany) and analyzed by Odyssey software (UK).

2.9. Statistical analysis

Data analysis was performed using SPSS version 11.5. Individual groups were assessed with one-way ANOVA and Tukey post hoc test. Differences were considered significant at p < 0.05.

3. Results

3.1. Carboxyhemoglobin (COHb) levels after CO intoxication

Carboxyhemoglobin values are shown in Table 2. The COHb level is a biochemical marker of CO poisoning severity. High level of COHb was shown in 3000 ppm CO group.

3.2. EPO decreased CO-induced myocardial apoptosis

Apoptosis cell death is expected in cardiac ischemia. In addition, is-
chemic injury occurs due to CO intoxication. In this study, to evaluate whether the effect of CO and EPO administration on myocardial apoptosis, in situ assay of DNA fragmentation was performed and activity of caspase-3 was measured.

As TUNEL results showed, intoxication by 3000 ppm and 1000 ppm CO significantly increased TUNEL-positive cells to 17.6% (p < 0.001) and 11.11% (p < 0.001) respectively compared with the control (1.4%). The increase in positive stained cells in the 250 ppm CO group was not sig-
nificant in comparison to the control (from 1.4% to 2.4%). EPO admin-
istration after 3000 ppm CO intoxication significantly reduced the percentage of apoptotic cells to 11.11% (3000 ppm CO + EPO vs. 3000 ppm CO, p < 0.001), also EPO administration after 1000 ppm CO decreased apoptotic index significantly to 6% in comparison to the untreated 1000 ppm CO group (p < 0.01) (Fig. 1).

Caspase-3 is a key factor which implicated as a downstream caspase in the apoptosis pathway. The effect of EPO on CO-induced apoptosis was further investigated by evaluation of caspase-3 activity in all groups. As shown in Fig. 2, intoxication by 3000 ppm CO significantly in-
creased caspase-3 activity to 9.1 fold when compared with the control group (p < 0.001). EPO treatment effectively prevented the increase of caspase-3 activity (3000 ppm CO + EPO vs. 3000 ppm CO, p < 0.001) which indicated its inhibitory effect on caspase-3. No significant increase in caspase-3 activity was shown following intoxication by 1000 and 250 ppm of CO in comparison with the control (Fig. 2).

3.3. EPO decreased CO-induced MMP disruption

MMP is necessary for cell viability as MMP disruption results in apo-
ptosis. To determine whether CO and EPO influence MMP, the CO expo-
sure groups of 3000 ppm, with or without EPO administration, where the most detrimental effects were observed, were chosen. Isolated mi-
tochondria from heart tissues were incubated with JC-1 as a MMP sen-
sitive dye. Since the uptake of the cationic carbocyanine dye (JC-1) into the mitochondrial matrix depends on membrane potential, JC-1 con-
centrates in the matrix of healthy mitochondria to form J-aggregates and emits red/orange fluorescence in contrast to JC-1 monomer which emits green fluorescence. MMP was measured in the 3000 ppm CO and also the EPO receiving group. The results were compared with the controls (Figs. 3, 4). The control group exhibited a normal pattern of mitochon-
dria with high fluorescence (3000 ppm vs. control, p < 0.0001). There was no significant difference between the 3000 ppm CO and the valinomycin positive control group. EPO treatment resulted in a significant increase in the fluorescence intensity as compared to the untreated-3000 ppm CO group (3000 ppm CO + EPO vs. 3000 ppm CO, p < 0.001).

<table>
<thead>
<tr>
<th>Groups</th>
<th>Mean ± SD</th>
<th>Range (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3000 ppm groups</td>
<td>70 ± 8</td>
<td>60–76</td>
</tr>
<tr>
<td>1000 ppm groups</td>
<td>31 ± 11</td>
<td>19–46</td>
</tr>
<tr>
<td>250 ppm groups</td>
<td>10 ± 5</td>
<td>13–10</td>
</tr>
<tr>
<td>Control group</td>
<td>1 ± 0.9</td>
<td>0.7–1.5</td>
</tr>
</tbody>
</table>

Table 2

Blood carboxyhemoglobin levels after CO poisoning in animals.
3.4. Western blot results

Bcl2 family has roles in regulation of apoptosis and mitochondrial membrane potential. To address whether CO intoxication and EPO treatment influence the Bcl2 family proteins, the immunoblotting of Bax and Bcl2 were assessed at the 3000 ppm CO group, where the most cardiotoxic CO effects were noticed, and at 3000 ppm CO + EPO group compared to control groups (Fig. 5). Our results showed that 3000 ppm CO poisoning decreased Bcl2/Bax ratio significantly compared with the control (p < 0.01), while EPO significantly preserved this ratio (3000 ppm CO + EPO vs. 3000 ppm CO, p < 0.01).

Fig. 1. Quantitative analysis of TUNEL staining. Apoptotic index represents TUNEL positive nuclei. Apoptotic index was significantly higher in the 3000 and 1000 ppm CO groups in comparison with the control (**p < 0.001 vs. control), EPO administration following CO intoxication significantly reduced TUNEL positive cells in these groups (**p < 0.001 & †p < 0.01, vs. untreated group). Values are expressed as mean ± SEM.

Fig. 2. Effect of EPO and CO on caspase-3 activity. Caspase-3 activity increased significantly by 3000 ppm CO compared to the control (**p < 0.001, vs. control). This was reduced by EPO treatment (***p < 0.001, vs. 3000 ppm CO group). Values represent mean ± SEM.

Fig. 3. Quantitative analysis of CO poisoning and EPO effect on mitochondria membrane potential (ΔΨm). 3000 ppm CO intoxication resulted in decreased red fluorescence intensity (8.25 ± 0.5 vs. 62.32 ± 5.2, ***p < 0.001). EPO treatment effectively improved fluorescence intensity in FL1 compared to the untreated group (**p < 0.001, vs. 3000 ppm CO group). For the positive control group, isolated mitochondria were treated with valinomycin. Results are mean ± SEM.

4. Discussion

Acute or chronic exposure to CO confers an increased risk for the cardiovascular system ranging from cardiac arrhythmias to myocardial injury, fibrosis and cardiomyopathy [5, 10]. Exposure to higher concentrations of exogenous CO, acutely or chronically via air pollution can exacerbate outcomes of cardiovascular disease [23], despite the fact that at very low concentrations of CO (nanomoles), especially by using carbon monoxide-releasing molecules (CORMs), anti-apoptotic effects are observed. Recent findings on animal models suggest that chronic CO exposure via atmospheric air pollution promotes cardiac hypertrophy, elevates basal heart rate and leads to impaired contractility and spontaneous arrhythmias [24]. On the other hand, while apoptosis is a mechanism for eliminating redundant cells, in the same time is a key factor in the pathogenesis of heart diseases, including heart failure. Other studies have documented apoptosis occurrence in the nervous tissue due to CO poisoning [10, 25]. However, there is little data on the role of apoptosis in CO-induced cardiac injury.

The results of the present study revealed the occurrence of myocardial apoptosis following exposure to 1000 ppm and even more significantly to 3000 ppm CO in rats. However there was no statistically significant difference in apoptosis occurrence between the 250 ppm CO group and the control.

The induction of apoptosis is associated with the activation of aspartate specific cysteine proteases, including caspase-3. In this aspect mitochondria may play an important role in apoptosis by releasing cytochrome c and activating caspase-9, which activates caspase-3, the molecule responsible for DNA cleavage. Caspase-3, as a common component of apoptotic signaling, mediates both mitochondria dependent and death receptor-dependent apoptosis pathways [26]. CO exposure in the present study at 3000 ppm led to a 9 fold increase of caspase-3 levels compared to controls. It is interesting though that CO exposure to 1000 ppm was able to significantly increase TUNEL-positive cells at 11.11% of rat myocardium, while no significant caspase-3 elevation was noticed, possibly indicating the difference in sensitivities between the two methods and different time course of DNA cleavage and
changes in caspase-3 activity. Caspase-3 activity is increased in the early stages of apoptosis, while DNA cleavage takes place later in the process.

During CO poisoning, tissue hypoxia, impaired cellular respiration and consequently stress responses such as production of reactive oxygen species (ROS) lead to cell injury resulting in neuronal and cardiac insults [25]. It is well known that CO is cardio-toxic and myocardial ischemia occurs as a result of sub lethal acute CO intoxication [6, 27]. According to our previous study, EPO suppressed ischemic changes demonstrated in electrocardiogram recordings which was induced by CO poisoning in rat [19]. It is well known that apoptosis can occur during hypoxic/ischemic conditions [28]. Since anti-apoptotic effects play a role in the EPO anti-ischemic property [12], the EPO effect on CO-induced myocardial apoptosis was studied. The results of the present study demonstrated that administration of 5000 IU/kg EPO after CO intoxication, effectively reduced TUNEL-positive cells and caspase-3 activity as compared to the control. Cai et al., who examined the effect of 5000 IU/kg EPO 24 h prior to cardiac ischemia in rat, have shown the EPO cardio-protective effect as well as an improvement of the heart function. Their results indicated reduction in number of TUNEL-positive cells and caspase-3 activity [29]. According to in vivo studies, EPO administration at the onset or after ischemia could reduce apoptosis and improve cardiac function [30, 31]. Moon and colleagues have pointed out that a single dose (3000 IU/kg) of EPO was enough for protection against cardiac ischemia compared to repeated doses in rat. In addition, they have described that EPO administration even after permanent ligation still had cardio protective effect [32]. Their results have shown that EPO continued to reduce myocardial apoptosis 1, 4 and 8 weeks after infarction. Such reduction reached 50% at 24 h, while 8 weeks after EPO administration still accounted for 15–25% compared to the controls. In the present study, an EPO protective effect on CO-induced myocardial injury was evident through its anti-apoptosis property.

Mitochondrial injury occurs during apoptosis that include mitochondrial membrane depolarization, loss of mitochondrial oxidative phosphorylation and release of cytochrome c. However, some studies have described that loss of the MMP may be an early event in the apoptosis pathway [33, 34]. The results of the present study showed that CO intoxication induced mitochondrial membrane depolarization significantly in the myocardial cells, while EPO administration increased MMP to 4.7 fold compared to the control. It’s believed that EPO antioxidant property has implicated to its anti-apoptotic and protective effects [35]. Since the role of oxidative stress in CO poisoning, the EPO protective effect on CO cardiotoxicity in this study may be related to its anti-oxidant property. It has also been suggested that EPO may exert a direct protective effect at least through anti-apoptotic and anti-degenerative activity independent of its erythropoietic effect [36].

Apoptosis is regulated by various apoptosis-related proteins. Bcl-2 protein acts as an anti-death factor, preventing the release of cytochrome c and other apoptogenic factors from mitochondria [37] while activation of Bcl2 family proteins is considered to be an upstream regulator of the MMP. On the other hand, Bax proteins reduce MMP and thereby cause cytochrome c release and caspase activation, which leads to apoptosis. Our results showed that EPO improved Bcl2/Bax ratio which was reduced by CO exposure at 3000 ppm.

Fig. 4. Histogram of mitochondria membrane potential using JC-1 fluorescence intensity in FL1. Effect of (A) valinomycin (as positive control), intoxication by 3000 ppm CO (B) and EPO treatment (C) on changes from control of median fluorescence.
References


