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Simulated urban carbon monoxide air pollution exacerbates rat heart ischemia-reperfusion injury

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MYOCARDIAL DAMAGES RESULTING from ischemia-reperfusion (I/R) are recognized to be the result of a complex interplay between genetic and environmental factors. Epidemiological studies suggested that, among environmental factors, carbon monoxide (CO) urban pollution can be linked to cardiac diseases and mortality. The aim of this work was to evaluate the impact of exposure to CO pollution on cardiac sensitivity to I/R. Regional myocardial I/R was performed on isolated perfused hearts from rats exposed for 4 wk to air enriched with CO (30–100 ppm). Functional variables, reperfusion ventricular arrhythmias (VA) and cellular damages (infarct size, lactate dehydrogenase release) were assessed. Sarcomere length shortening and Ca2+ handling were evaluated in intact isolated cardiomyocytes during a cellular anoxia-reoxygenation protocol. The major results show that prolonged CO exposure worsens myocardial I/R injuries, resulting in increased severity of postischemic VA, impaired recovery of myocardial function, and increased infarct size (60 ± 5 vs. 33 ± 2% of ischemic zone). The aggravating effects of CO exposure on I/R could be explained by a reduced myocardial enzymatic antioxidant status (superoxide dismutase −45%; glutathione peroxidase −49%) associated with impaired intracellular Ca2+ handling. In conclusion, our results are consistent with the idea that chronic CO pollution dramatically increases the severity of myocardial I/R injuries.

environmental pollution; myocardial infarction; antioxidant status

Among the environmental factors that could influence the development of cardiovascular diseases, several epidemiological studies have recently suggested that urban atmospheric pollution may exert adverse effects on cardiovascular health (7, 11, 12, 15). Among the numerous pollutants, carbon monoxide (CO) has been described as one of the main pollutants responsible for the development of cardiovascular diseases (9, 35). In urban environments, CO concentration usually varies from 2 to 40 ppm, but during heavy traffic it may be as high as 170 ppm (10, 34, 40). At this level, CO exposure has been correlated with hospital admissions, mortality, and morbidity related to cardiovascular diseases (9, 13, 29). Today, although the pathophysiological mechanisms regarding acute CO poisoning are well known, mechanisms associated with chronic exposure to lower concentrations of CO, consistent with urban environmental pollution, remain unclear. We (2) and Bye et al. (14) have recently reported that prolonged CO exposure induces a pathological myocardial cellular phenotype characterized by a major remodeling of the excitation-contraction coupling. Such deleterious consequences may worsen the effect of cardiovascular diseases.

The aim of this experimental work was to challenge the impact of a chronic exposure to simulated CO urban pollution on the sensitivity of the myocardium to I/R in a rat model. The major result shows that prolonged exposure to CO at a level found in the urban environment has a dramatic deleterious impact on the sensitivity of the myocardium to I/R.

METHODS

Experiments complied with the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health (National Institutes of Health Publications no. 85-23, revised 1996) and was approved by the French Ministry of Agriculture.

Animals and CO Exposure

Adult, male Wistar rats (n = 64; 345 ± 7 g; Charles River Laboratories) were randomly assigned to the following two groups: CO rats (exposed for 4 wk to simulated CO urban pollution, n = 32) and control animals (Ctrl rats, exposed to standard filtered air, n = 32). CO rats were housed in an airtight exposure container for 4 wk. Exposure was performed according to a 12:12-h CO in the air-ambient air cycle. To simulate CO urban pollution, exposure was performed as follows: during CO exposure, a CO concentration of 30 ppm was maintained in the airtight container and monitored with an aspirative CO analyzer (CHEMGARD Infrared Gas Monitor NEMA 4 Version;
Cardiomyocytes were transferred to a glass petri dish and placed in an anoxic chamber (O₂ level ~2%) for 60 min, followed by a 60-min reoxygenation in ambient air (O₂ ~19.4%). Unloaded cell shortening and Ca²⁺ concentration (indo 1 dye) were measured using field stimulation (0.5 Hz, 22°C, 1.8 mM external Ca²⁺) before and after anoxia-reoxygenation (A/R). Sarcomere length (SL) and fluorescence (405 and 480 nm) were recorded simultaneously (IonOptix system; Milton). The A/R experiment was then carried out in the presence or absence of a nonspecific antioxidant [N-acetylcystein (NAC), 20 μM].

Biochemical Assays

Heart antioxidant enzyme activity. A fourth set of rats (n = 6/group) was used to evaluate the enzymatic antioxidant status of the myocardium consecutively to chronic CO exposure and before I/R. After the end of CO exposure (24 h), the hearts were freeze-clamped, and the frozen ventricular tissue was homogenized in Tris-HCl buffer (60 mM Tris-HCl and 1 mM diethyliaminopentaacetic acid, pH 7.4, 10 ml/g wet wt) using a Teflon potter homogenizer. Tissue homogenates were then centrifuged for 10 min at 200 g at 4°C to remove all nuclear debris. Cardiac superoxide dismutase (SOD) activity was assessed in the supernatant according to the method described by Marklund (22). Cardiac glutathione peroxidase (GPs) activity was assessed spectrophotometrically on the cytosolic fraction according to the method described by Flohe and Günzler (18). Catalase activity was determined according to the method described by Beers and Sizer (8). All enzyme activities were expressed in units per milligram protein (U/mg protein). The modified method of Lowry et al. (21) was used to determine protein content of tissue homogenates, using BSA as standard.

Lactate dehydrogenase in coronary effluents. Lactate dehydrogenase (LDH) activity was measured in coronary effluents by spectrophotometry using an LDH kit (LDH-P; BIOLABO). Measurements were made at the end of stabilization and at 10, 30, and 60 min of reperfusion. LDH activity was normalized to coronary blood flow.

Thiobarbituric acid-reactive substances in LV tissues. Thiobarbituric acid-reactive substances (TBARS) were assessed in LV tissues after 30 min of reperfusion using the thiobarbituric acid test (TBA) test. Frozen heart tissue (120 mg) was homogenized in 1 ml 0.1% TCA solution. The homogenate was centrifuged at 12,000 g for 15 min, and 0.5 ml of the supernatant was added to 1 ml of 0.5% TBA in 20% TCA. The mixture was incubated in boiling water for 30 min, and the reaction was stopped by placing the reaction tubes in an ice bath. Tubes were briefly vortexed, triplicate 200-μl aliquots were taken from each tube and placed in 96-well plates, and the supernatant absorbance was read at 532 nm in a microplate reader. The value for nonspecific absorption at 600 nm was subtracted. The amount of TBARS (red pigment) was calculated using an extinction coefficient of 155 mM⁻¹-cm⁻¹.

Statistics

Data were analyzed using one-way ANOVA between groups and repeated-measures ANOVA when necessary. When significant interactions were found, a Tukey-Kramer test was applied. The distribution of the hearts based on the various arrhythmic scores was analyzed by a nonparametric Mann-Whitney U-test. Bonomially distributed variables (such as incidence of VF) were analyzed using nonparametric Yates’ chi square test (Statview; Adept Scientific, Letchworth, UK). A level of P < 0.05 was considered statistically significant. Data are expressed as group means or group mean fractions of baseline ± SE.

RESULTS

Effects of CO Exposure on Myocardial Reperfusion Ventricular Arrhythmias

The time course of reperfusion-induced VPB, VT, and VF in individual hearts is shown in Fig. 1C. Figure 1D shows a
significant difference in the distribution of the arrhythmic scores observed in both experimental groups. According to the increased mean arrhythmic score observed in CO rats during reperfusion, the arrhythmia severity was higher in CO rats compared with Ctrl rats (Fig. 1E). Moreover, sustained VF were triggered more frequently in the CO rats compared with Ctrl rats (54 vs. 9%, \(P < 0.05\) nonparametric Yates’ chi square test).

**Effects of CO Exposure on Postischemic Recovery of Myocardial Function**

Any difference regarding cardiac function was reported between CO and Ctrl rats before ischemia (Table 1). Postischemic recovery of LV developed pressure and contractility, assessed during reperfusion, was significantly lower in CO rats compared with Ctrl (Fig. 2). Indeed, the postischemic recovery of LV developed pressure, \(+dP/dt_{\text{max}}\), and \(-dP/dt_{\text{max}}\) were significantly altered in the CO rats compared with Ctrl rats (Fig. 2, A–C).

These functional results were paired with some deleterious effects of CO exposure on postischemic myocardial coronary blood flow recovery (Fig. 2D). Indeed, although no difference in coronary blood flow was reported between CO and Ctrl rats before ischemia (Ctrl rats: \(11.5 \pm 0.9\) ml/min vs. CO rats: \(11.8 \pm 1.4\) ml/min), coronary blood flow was significantly lower in CO rats compared with Ctrl rats during reperfusion.

**Effects of CO Exposure on Myocardial Postischemic Reperfusion-Induced Cellular Death**

The deleterious effects of CO exposure on I/R-induced injury were characterized by an increase in myocardial infarct...
The infarct size was markedly increased in CO rats (60 ± 5 vs. 33 ± 2% of the risk zone; P < 0.05). LDH released in coronary effluents, used as an index of cell death, was significantly augmented at the onset of reperfusion in both Ctrl and CO rats (1.91 ± 0.30 to 5.84 ± 1.45 U/min for Ctrl rats; 2.69 ± 0.54 to 14.26 ± 3.20 U/min for CO rats; P < 0.05). Moreover, the peak of LDH release at the onset of reperfusion was significantly higher in CO rats than in Ctrl rats. Finally, LDH release remained significantly higher in CO rats after 30 and 60 min of reperfusion. No difference regarding LDH release was observed during the stabilization period (Fig. 3C). LV TBARS concentration was significantly increased in CO compared with Ctrl rats after 30 min of reperfusion (6.43 ± 0.76 vs. 4.40 ± 0.35 nmol/g; Fig. 3D).

Table 1. Myocardial function during regional ischemia-reperfusion protocol in Ctrl and CO rats

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>30 min of Ischemia</th>
<th>5 min of Reperfusion</th>
<th>30 min of Reperfusion</th>
<th>60 min of Reperfusion</th>
<th>90 min of Reperfusion</th>
<th>120 min of Reperfusion</th>
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<tbody>
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<td></td>
<td>LVDP, mmHg</td>
<td>+dP/dtmax, mmHg/s</td>
<td>–dP/dtmin, mmHg/s</td>
<td>LVDP, mmHg</td>
<td>+dP/dtmax, mmHg/s</td>
<td>–dP/dtmin, mmHg/s</td>
<td>LVDP, mmHg</td>
</tr>
<tr>
<td>Ctrl rats</td>
<td>91.79 ± 7.47</td>
<td>3.365 ± 304</td>
<td>−1.944 ± 123</td>
<td>65.50 ± 4.82</td>
<td>2.304 ± 487</td>
<td>−1.447 ± 199</td>
<td>63.31 ± 5.15</td>
</tr>
<tr>
<td>CO rats</td>
<td>93.85 ± 12.11</td>
<td>2.932 ± 369</td>
<td>−1.994 ± 219</td>
<td>53.29 ± 5.66</td>
<td>1.757 ± 240</td>
<td>−1.11 ± 162</td>
<td>43.71 ± 5.66*</td>
</tr>
</tbody>
</table>

Values are means ± SE; LVDP, left ventricular developed pressure; +dP/dtmax, maximal derivative of left ventricular pressure; –dP/dtmin, minimal derivative of left ventricular pressure; Ctrl rats, rats exposed to filtered standard air; CO rats, rats exposed to carbon monoxide. *P < 0.05 vs. Ctrl rats.

At the cellular level, prolonged CO exposure induced impairments of ventricular myocyte function (Fig. 4). Indeed, before A/R, SL shortening (Fig. 4, A and B) as well as Ca2+ transient (Fig. 4, C and D) were significantly impaired in CO rats compared with Ctrl rats. In addition, diastolic cytosolic Ca2+ was markedly higher in CO compared with Ctrl rats (Fig. 4E). Consecutively to A/R, SL shortening was decreased in both groups (Fig. 4, A and B) and remained significantly lower in CO compared with Ctrl rats. This decreased SL shortening was associated with a decreased Ca2+ transient in both groups consecutive to A/R. It is of note that Ca2+ transient from CO rats remained lower than Ca2+ transient from Ctrl rats (Fig. 4, C and D). Following A/R, diastolic Ca2+ increased in the two experimental groups; however, for this parameter, statistical significance was reported only in Ctrl rats. Consequently,

Fig. 2. Effects of CO exposure on the time course of changes in left ventricular function parameters during a 30-min ischemia and 120 min of reperfusion. Change in left ventricle developed pressure (A), maximal (+dP/dtmax, B), and minimal (–dP/dtmin, C) derivative of left ventricular pressure over time, and coronary blood flow (D). Data are presented as mean fraction of baseline ± SE (n = 6 rats/group, repeated-measures ANOVA, *P < 0.05, Ctrl vs. CO rats).
following A/R, no difference was observed concerning intracellular diastolic Ca\(^{2+}\) between Ctrl and CO rats (Fig. 4E).

Following A/R, the incubation of NAC (a nonspecific antioxidant) reduced the impairment of cardiac cell contraction in both Ctrl and CO rats. In addition, NAC infusion partly blunted the higher sensitivity of CO rat cardiomyocytes to A/R, since in this condition no difference was reported between Ctrl and CO groups regarding SL shortening (Fig. 5A). As far as the alterations of Ca\(^{2+}\) handling are concerned, the incubation of NAC improved Ca\(^{2+}\) transient in both Ctrl and CO rats. However, the difference between the two experimental populations was still reported (Fig. 5B). Finally, diastolic cytosolic Ca\(^{2+}\) was significantly reduced in CO rat cardiomyocytes in the presence of NAC compared with the corresponding controls (Fig. 5C). In the presence of NAC, no difference was reported regarding this variable between Ctrl and CO rats.

**Effects of CO Exposure on Myocardial Antioxidant Enzyme Activity**

In CO rats, following 4 wk of exposure to simulated urban CO pollution, cardiac SOD and GPX activities were significantly lower compared with Ctrl rats (Fig. 6, A and B). No significant change in catalase activity was observed in this model of CO exposure (Fig. 6C).

**DISCUSSION**

To the best of our knowledge, our study is the first to investigate the effects of prolonged exposure to simulated urban environmental CO pollution on myocardial sensitivity to I/R. The major result is that prolonged exposure to simulated urban CO pollution worsens myocardial I/R injuries, promoting a major increase in the severity of arrhythmic events, an impairment of myocardial function observed at both global and cellular levels, and an increase in the infarct size. These phenomena could be mainly related to hidden effects of CO exposure on myocardial phenotype, including 1) an impairment of cellular Ca\(^{2+}\) handling and 2) an altered antioxidant status of the myocardium.

**CO Exposure and Myocardial I/R**

Eventhough CO urban pollution has been associated with increased cardiovascular disease and cardiac mortality (9, 13, 29), we demonstrate here that chronic CO exposure renders the heart more vulnerable to I/R. Previous experimental studies have only documented the effect of acute CO exposure or to CO-releasing molecules used as preconditioning strategies to protect the myocardium against I/R injury (3, 6, 19). It was also reported that endogenous CO production could protect the heart from I/R injuries. These beneficial effects of endogenous
CO production during I/R could be because of a decrease in oxidative stress (26), an enhanced Ca\(^{2+}\)/H\(_{11001}\) handling (36), and CO anti-inflammatory and anti-apoptotic properties (20), which may ultimately lead to a decrease in I/R-induced VF (4, 5). In our model, no difference regarding cardiac function and coronary blood flow was evidenced between Ctrl and CO rats before I/R. Remarkably, only postischemic reperfusion allowed us to distinguish the hearts of CO-exposed rats from those of Ctrl rats, thereby pointing out the deleterious effect of CO. These results are in line with our previous study (2), which exhibited no difference of cardiac function in the basal condition but highlighted functional impairments in CO rats in stressful conditions (i.e., \(\beta\)-adrenergic stimulation). Therefore, discrepancies between our results and studies reporting the protective role of CO exposure could be explained by differences in the duration and severity of CO exposure (3, 6, 19). In our model, CO rats experimented a chronic (4 wk with 12 h daily exposure) CO exposure mimicking urban concentrations (30–100 ppm). In addition, it has to be noted that, in our model, to avoid the acute effects of CO on the myocardium, rats were housed for 24 h in standard filtered air before I/R study. Therefore, under our experimental conditions, no difference of carboxyhemoglobin was made obvious between Ctrl and CO rats at the time of the I/R procedure (carboxyhemoglobin 24 h after exposure, CO rats: 1.2 ± 0.4% vs. Ctrl rats 1.0 ± 0.5%; not significant).

**Effects of CO Exposure on Postischemic Myocardial Injuries**

The higher susceptibility of the myocardium to I/R in CO-exposed rats was notably characterized by higher propensity to arrhythmic events at the onset of reperfusion. Higher arrhythmic score and higher occurrence of sustained VF (54 vs. 9%) were evidenced. Arrhythmias, and more particularly sustained VF, are the most dangerous consequences of myocardial I/R, since they induce an impairment of blood circulation and ultimately lead to sudden cardiac death. Sheps et al. (31, 32) have already highlighted the proarrhythmic effect of CO exposure (1 day at 100 or 200 ppm) in patients with documented coronary artery diseases. However, as mentioned above, in our model, no difference of carboxyhemoglobin was reported between Ctrl and CO rats.
at the time of the I/R procedure, avoiding then the acute effects of CO on the myocardium. In CO rats, the higher sensitivity of the myocardium was also characterized by an impairment of postischemic recovery of myocardial function. This could be related to the significantly impaired cellular contractile function observed, consecutive to A/R, on isolated cardiomyocytes of CO rats compared with Ctrl rats. This result suggests that cardiomyocyte damages lead to a reduced postischemic recovery of cardiac function. It seems, however, that the main factor involved in the explanation of this result was that CO pollution is associated, in our model, with a marked increase of I/R-induced cardiac cell death. I/R injuries, including cardiomyocyte impairments as well as arrhythmic events, were mainly explained, in past literature, by increased oxidative stress and Ca\(^{2+}\)-handling alteration (25, 41).

**Implication of Ca\(^{2+}\) and Oxidative Stress**

The increased sensitivity to I/R of the myocardium after CO exposure was characterized by the increase of arrhythmic events, increased functional disturbances, and cell death. The cellular mechanisms underlying these aspects of the reperfusion syndrome may involve impairments of Ca\(^{2+}\) handling or overproduction of oxygen-derived free radicals. In a recent study, we have shown that CO pollution by itself initiates a pathological phenotype of the cardiomyocytes involving a profound remodeling of the excitation-contraction coupling through Ca\(^{2+}\)-handling alteration (2), which was confirmed in the present work. This remodeling was characterized by an impairment of cardiomyocyte shortening that was not observed at the whole heart level. This phenomenon, which has already been reported by our team, may involve compensatory mechanisms (2) that remain to be investigated. The cardiomyocytes also exhibit an increase in diastolic Ca\(^{2+}\) resulting from an altered Ca\(^{2+}\) reuptake in the sarcoplasmic reticulum (SR) due to a decreased sarco-(endo)plasmic reticulum Ca\(^{2+}\)-ATPase (SERCA) 2a expression and also potentially from a Ca\(^{2+}\) leak from the SR through the ryanodine receptors (2). Alterations of Ca\(^{2+}\) handling may be involved in cardiomyocyte dysfunction and ultimately in cell death during I/R (1, 33, 38). It is therefore very likely that these alterations, associated with CO exposure, increase the severity of cardiomyocytes and whole heart injuries related to I/R. Indeed, in our study, at the whole heart level, CO exposure increases cardiomyocyte death after I/R. Considering only viable myocytes, we report here that SL shortening and Ca\(^{2+}\) handling are altered by A/R to the same extent in both Ctrl and CO rats, resulting therefore in more pronounced dysfunctions in CO rats compared with Ctrl rats. Therefore, it seems that Ca\(^{2+}\) handling in viable cardiomyocytes was not more impaired by A/R in CO rats, but that the lower amplitude of Ca\(^{2+}\) transient reported in this population following A/R was the result of prolonged CO exposure-induced phenotypical changes. Consequently, a major finding of the present study was that the impaired Ca\(^{2+}\) handling observed before to A/R is a key factor in the development of functional impairments and arrhythmic events observed at the onset of reperfusion in CO rats and is therefore a candidate to explain higher sensitivity of CO rat myocardium to I/R.

The essential role of oxidative stress in the pathogenesis of myocardial I/R injury has been largely reported (16, 30, 41). Indeed, exacerbated oxidative stress during I/R is a key factor in the worsening of postischemic arrhythmias, cardiac dysfunction, and irreversible cardiomyocyte damages (16, 17, 25, 41). Oxidative stress is defined as an imbalance between the pro-
duction of reactive oxygen species and biological antioxidant systems that are involved in the detoxification of reactive intermediates. An important result of our study is the impaired enzymatic antioxidant status of the myocardium, including a major decreased activity of SOD (−45%), GPx (−49%), and catalase (−26%, not statistically significant). We previously discussed the potential role of altered redox status of the myocardium to explain the effects of prolonged exposure to CO on excitation-contraction coupling (2). Indeed, since proteins involved in Ca^{2+} handling are potential targets for redox alterations, decrease of antioxidant defenses associated with the increased activity of thioredoxin reductase observed in our previous study (2) confirmed a CO-induced oxidative stress. Therefore, those alterations of enzymatic antioxidant defense could play a major role in phenotypical changes of CO-exposed rat myocardium, mainly affecting Ca^{2+} homeostasis. Besides, SOD prevents changes in myocardial function, and Ca^{2+} homeostasis in isolated hearts subjected to I/R (27), catalase, and GPx plays a major role in protecting the myocardium from I/R injury (16). We have observed an increase in TBARS production, used as a marker of lipid peroxidation, and therefore oxidative stress, in CO-exposed rats following I/R. Therefore, we can postulate that the lower enzymatic antioxidant defense that was observed consecutively to CO exposure could contribute to the increased sensitivity of the myocardium to I/R damages. To prevent oxidative stress-induced alterations, an acute antioxidant strategy, using a nonspecific antioxidant (NAC), was performed during cellular A/R. This acute antioxidant strategy was found able to prevent the deleterious effects of CO exposure on SL shortening during A/R. This result highlights the implication of an increased cardiac oxidative stress in our model of CO-exposed rats. However, the deleterious effects of CO exposure on Ca^{2+} transient were not prevented by this acute antioxidant strategy. This observation is not surprising, since prolonged exposure to CO pollution results in phenotypical remodeling of Ca^{2+}-handling proteins, such as decreased SERCA2a expression (2). This profound remodeling of the Ca^{2+}-handling phenotype, which was reported in CO rat myocardium, could not be reversed by such an acute antioxidant strategy. Therefore, chronic antioxidant therapy, notably by preventing myocardial phenotypical changes, could be a promising strategy for protecting the heart against the deleterious effects of chronic CO exposure on myocardial sensitivity to I/R.

In conclusion, this study shows that chronic exposure to CO consistent with air pollution from the urban type significantly increases the effects of a myocardial infarction in rats and could be considered as a major health risk. Indeed, the World Health Organization estimates that air pollution is responsible for 800,000 premature deaths worldwide each year, and, particularly, exposure to air pollution increases the risk of mortality from cardiovascular disease by 76% (23). Among the numerous pollutants, CO has been described as one of the main pollutants responsible for the development of cardiovascular diseases (9, 35). Prolonged CO exposure worsens I/R-linked cardiac injuries and therefore provides an experimental rationale to explain the increased risk of cardiac mortality observed in exposed populations. In summary, prolonged CO exposure-induced cardiac phenotypical changes, such as an imbalance in the cardiomyocyte oxidant status and an impairment of Ca^{2+} handling, are likely to predispose the heart to I/R injuries.

GRANTS
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DISCLOSURES
No competing financial interests exist.

Fig. 6. Effects of CO exposure on superoxide dismutase (SOD, A), glutathione peroxidase (GPx, B), and catalase (C) activities expressed in mU/mg protein. Data are presented as means ± SE (n = 6 rats/group, 1-way ANOVA, *P < 0.05, Ctrl vs. CO rats).

Aerobic capacity and cardiac contractility and induce pathological hyper-trophy.

Wisloff U.


Pollution and hospitalization for cardiorespiratory diseases.

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