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Carbon Monoxide Pollution Promotes Cardiac Remodeling and Ventricular Arrhythmia in Healthy Rats

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Rationale: Epidemiologic studies associate atmospheric carbon monoxide (CO) pollution with adverse cardiovascular outcomes and increased cardiac mortality risk. However, there is a lack of data regarding cellular mechanisms in healthy individuals.

Objectives: To investigate the chronic effects of environmentally relevant CO levels on cardiac function in a well-standardized healthy animal model.

Methods: Wistar rats were exposed for 4 weeks to filtered air ($\text{CO} < 1 \text{ ppm}$) or air enriched with CO (30 ppm with five peaks of 100 ppm per 24-h period), consistent with urban pollution. Myocardial function was assessed by echocardiography and analysis of surface ECG and *in vitro* by measuring the excitation-contraction coupling of single left ventricular cardiomyocytes.

Measurements and Main Results: Chronic CO pollution promoted left ventricular interstitial and perivascular fibrosis, with no change in cardiomyocyte size, and had weak, yet significant, effects on *in vivo* cardiac function. However, both contraction and relaxation of single cardiomyocytes were markedly altered. Several changes occurred, including decreased Ca^{2+} transient amplitude and Ca^{2+} sensitivity of myofilaments and increased diastolic intracellular Ca^{2+} subsequent to decreased SERCA-2a expression and impaired Ca^{2+} reuptake. CO pollution increased the number of arrhythmic events. Hyperphosphorylation of Ca^{2+} -handling and sarcomeric proteins, and reduced responses to β -adrenergic challenge were obtained, suggestive of moderate CO-induced hyperadrenergic state.

Conclusions: Chronic CO exposure promotes a pathological phenotype of cardiomyocytes in the absence of underlying cardiomyopathy. The less severe phenotype *in vivo* suggests a role for compensatory mechanisms. Arrhythmia propensity may derive from intracellular Ca^{2+} overload.

Keywords: heart failure; atmospheric pollution; Ca^{2+} handling; protein kinase A pathway

The World Health Organization estimates that 3 million people are killed annually worldwide by outdoor air pollution caused by vehicles and industrial emissions (<http://www.who.int>; <http://www.infoforhealth.org>). Notably, air pollution increases the risk of mortality from cardiovascular disease by 76% (1) with deaths related to ischemia, ventricular arrhythmia (VA), and heart

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Most studies of the effects of atmospheric pollution provide correlations between air quality and cardiovascular outcomes, but do not provide mechanistic information owing to technical limitations in human subjects. Our findings provide a rationale at the cellular and molecular levels in support of epidemiologic studies pointing to the deleterious effects of carbon monoxide pollution on cardiac function.

What This Study Adds to the Field

This study shows that the deleterious effects of chronic carbon monoxide exposure may be barely detectable. However, even in the absence of underlying cardiopathy, cardiomyocytes may develop a pathological phenotype with some features commonly observed at the early stages of heart failure.

failure (HF) (2–4). Among the various agents that constitute the mix of atmospheric pollution, carbon monoxide (CO) is a ubiquitous pollutant found in many common sources (secondhand smoke, vehicular exhaust, industrial emissions, etc.). Outdoor CO exposure is closely associated with hospital admissions for cardiovascular disease (5) as well as cardiac mortality (6). In addition, CO pollution increases HF risk factors, such as blood viscosity and arrhythmia (7), and decreases heart rate variability (8, 9). However, studies investigating the effects of urban air pollution in humans are mainly restricted to epidemiological studies that only establish correlations between air quality and cardiovascular outcome. In this context, it is particularly difficult to discriminate the pathophysiological effects of various major atmospheric pollutants, such as CO, particulate matter, or lead. In addition, the mechanisms underlying the effects of chronic CO exposure on the heart have not been well established. In this regard, the use of an animal model has several advantages, including better standardization of experimental approaches and insights into cellular mechanisms.

The aim of our work was to investigate the effects of chronic CO exposure, under conditions mimicking urban air pollution, on rat cardiac function both *in vivo* and at the cardiomyocyte level. Our principal results were that chronic CO pollution initiated a pathological phenotype characterized by serious remodeling of the excitation-contraction coupling (ECC) of cardiomyocytes, with major alterations of Ca^{2+} handling leading to increased risk of VA.

METHODS

For a detailed description, see METHODS in the online data supplement. Experiments were performed with approval of the institutional animal care and use committee.

Animal Model and Chronic CO Exposure

In urban areas, ambient CO usually varies between 2 and 40 ppm, but under certain circumstances, such as during heavy traffic, its concentration may reach levels as high as 150–200 ppm (10, 11). In our study, male Wistar rats (13 wk old, n = 35) were exposed to CO in an airtight exposure container over 4 weeks. Rats were exposed to a basal CO concentration of 30 ppm during a 12-hour period completed with five peaks of 100 ppm (1 h each), to reproduce environmentally relevant variations in air quality (schema included in supplemental data). For the remaining 12 hours, the CO-exposed group of rats was exposed to filtered air (<1 ppm CO). The CO content was continuously monitored with an infrared aspirative CO analyzer (CHEM GARD, NEMA 4 Version, MSA, Greer, SC). Rats exposed to CO pollution were compared with rats exposed to filtered ambient air (CO <1 ppm; control, n = 25). Experiments were performed 24 h after the last CO exposure to avoid acute CO effects. Carboxyhemoglobin level measured 24 hours after the last CO exposure (1.2 ± 0.4%) did not differ from that of the control group (1.02 ± 0.22%) and was consistent with levels reached in human population exposed to high atmospheric pollution (12, 13).

In vivo Cardiac Investigation

Doppler echocardiography was performed to evaluate cardiac function and morphology (14). Hemodynamic evaluation (arterial and ventricular pressures) was performed in intact closed-chest anesthetized rats 24 hours after echocardiography (14). Measurements were performed before and after a β-adrenergic challenge by isoproterenol perfusion (1 mg/kg/min). Surface ECG recordings (Dataquest; Data Sciences International, Transoma Medical, Arden Hills, MN) were performed for a 24-hour period after the CO exposure protocol. Spontaneous rhythmic disorders, heart rate variability, mean, and standard deviation of all normal RR intervals were calculated from ECGs (24 h).

Histology

The quantification of interstitial fibrillar collagen content was performed on Sirius red-stained sections using morphometry (Lucia digital image analysis software; Nikon, Melville, NY).

Cardiac ECC Analysis

Ca²⁺ transient and cell fractional shortening. Single ventricular myocytes were isolated by enzymatic digestion as previously described (15). Unloaded cell shortening and Ca²⁺ concentration (Indo-1 dye) were measured using field stimulation (0.5 Hz, 22°C, 1.8 mM external Ca²⁺). Sarcomere length (SL) and fluorescence (405 and 480 nm) were simultaneously recorded (IonOptix system, Milton, MA) under basal conditions and after β-adrenergic stimulation with isoproterenol (100 nM).

Action potentials, L-type Ca²⁺ current, and K⁺ currents. Whole-cell patch-clamp experiments were performed (22 ± 2°C) with an Axopatch 200B (Axon Instruments, Burlingame, CA) (16). Currents were normalized to cell membrane capacitance (Cm) and expressed as densities. Because of the heterogeneity in action potential (AP) duration across the left ventricular (LV) free wall (17), we separated subepicardial and subendocardial cells.

Force measurements in permeabilized cardiomyocytes. Isometric force was measured in single permeabilized cardiomyocytes (18). Force was normalized to the cross-sectional area measured by imaging (IonOptix system). The relationship between Ca²⁺ activated force and internal Ca²⁺ concentration was measured at 2.3 μm SL and was fitted with a modified Hill equation. In some experiments, permeabilized cardiomyocytes were incubated with a recombinant catalytic subunit of protein kinase A (PKA, Sigma, St. Quentin, France) for 50 min at room temperature (19).

Protein Analysis

Ca²⁺ handling. Proteins were separated using 2 to 20% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Proteins were

revealed with specific antibodies and were expressed relative to calsequestrin content.

Myofilament proteins. Myofibrillar proteins were analyzed on skinned muscle strips from the LV (18). Phosphorylated and non-phosphorylated MLC-2 forms were detected with a cardiac MLC-2-specific antibody (Coger SA, Paris, France). TnI was revealed using specific antibodies for total cardiac TnI (Cat#4T21; Hytest, Turku, Finland) or the PKA-phosphorylated form of cardiac TnI (Cat#4T45; Hytest).

Cardiac Oxidative Status

Hearts (n = 6 per group) were freeze-clamped and the frozen ventricular tissue was homogenized in TRIS HCl buffer (TRIS HCl 60 mM, diethyltriaminopentaacetic acid 1 mM, pH 7.4, 10 ml/g wet wt.), using a Teflon Potter homogenizer. Tissue homogenates were then centrifuged (10 min at 20,000 × g at 4°C). Activities of total superoxide dismutase, catalase, and glutathione peroxidase, reflecting the antioxidant status, and thioredoxin reductase, reflecting the oxidant status, were measured in supernatants (20).

Statistical Analysis

Statistics were performed using StatView 5.0 (SAS Institute, Cary, NC). Data are presented as mean ± SEM unless otherwise specified. The effects of CO exposure, time, and/or β-adrenergic challenge were analyzed using one-way factorial analysis of variance or analysis of variance with repeated measures depending on the variables studied. Bonferroni *post hoc* tests were realized where appropriate. Statistical significance was defined as P less than 0.05.

RESULTS

Effects of CO Exposure on In Vivo Cardiac Morphology and Function

Chronic exposure to CO altered heart morphology and function. Rats from the CO group exhibited hypertrophic features, with increased heart weight/body weight as well as LV/body weight ratios. LV interstitial and perivascular fibrous tissue was

TABLE 1. EFFECTS OF CHRONIC CARBON MONOXIDE EXPOSURE ON CARDIOVASCULAR FUNCTION AND CARDIAC MORPHOLOGY IN VIVO

	Control (n = 12)	CO (n = 16)
Morphological data		
Body wt, g	514 ± 9	511 ± 5
Heart wt/100 g body wt, mg/100 g	259 ± 4	276 ± 6*
LV/100 g body wt, mg/100 g	204 ± 5	219 ± 4*
Fibrosis, % of total area	1.96 ± 0.29	3.72 ± 0.4*
Echocardiographic data		
LVEDD, mm	9.46 ± 0.14	9.61 ± 0.21
LVEsD, mm	5.76 ± 0.17	6.30 ± 0.16*
Anterior wall thickness, mm	1.02 ± 0.04	1.06 ± 0.06
Posterior wall thickness, mm	1.16 ± 0.11	1.29 ± 0.19*
LV shortening fraction, %	39.22 ± 1.18	34.35 ± 1.11*
Posterior wall end-systolic strain, %	98 ± 7	78 ± 9*
E/A-wave velocity ratio	1.89 ± 0.33	1.98 ± 0.34
Hemodynamic data		
Systolic arterial pressure, mm Hg	120 ± 10	121 ± 4
Diastolic arterial pressure, mm Hg	93 ± 9	96 ± 4
Mean arterial pressure, mm Hg	102 ± 10	105 ± 5
LV developed pressure, mm Hg	104 ± 5	106 ± 5
LV dP/dt max, mm Hg/s	5018 ± 326	4781 ± 255
LV dP/dt min, mm Hg/s	-4700 ± 285	-4676 ± 287
LV tau, s	8.22 ± 0.27	8.31 ± 0.16

Definition of abbreviations: CO = carbon monoxide; LV = left ventricular; LVEDD = left ventricular end-diastolic diameter; LVEsD = left ventricular end-systolic diameter; LV dP/dt max = maximal first derivative of left ventricular pressure; LV dP/dt min = minimal first derivative of left ventricular pressure; LV tau = time constant of left ventricular pressure decay; *P < 0.05 vs. control.

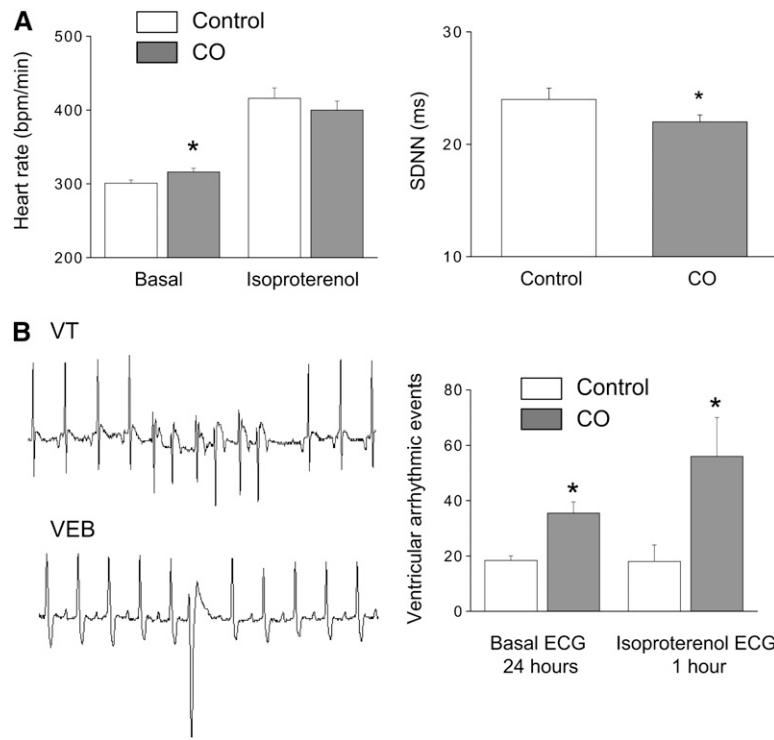


Figure 1. Analysis of *in vivo* arrhythmic events from ECG recordings and heart rate variability analysis in control and carbon monoxide (CO)-exposed rats. (A) Heart rate under basal conditions and after injection of isoproterenol (1 mg/kg i.p.) (right) and standard deviation of the normal RR intervals (left) ($n = 9$ rats in each group). (B) Typical examples of arrhythmic events occurring during 24-hour ECG recordings (left); analysis of the occurrence of arrhythmic events during 24-h measurements and after acute challenge with isoproterenol (average number of arrhythmic events over 1 h post-injection) (right). * $P < 0.05$. VEB = ventricular ectopic beat; VT = ventricular tachycardia

nearly doubled (Table 1; *see* Figure E2 in the online supplement). An increase in posterior wall thickness was observed, associated with a slight but significant global contractile dysfunction as indicated by a decrease in the LV shortening fraction and posterior wall end-systolic strain (Table 1). However, arterial blood pressure and LV hemodynamic parameters were similar in CO-exposed and control rats (Table 1). Taken together, our data suggested that chronic CO exposure has a weak, yet significant, effect on *in vivo* cardiac function.

Electrical Activity of Heart Measured *In Vivo* and Heart Rate Variability

Surface ECGs were recorded and analyzed in conscious rats from control and CO groups. Chronic CO exposure increased basal heart rate (Figure 1A) from 297 ± 2 bpm in control rats ($n = 8$) to 320 ± 8 bpm in CO-exposed rats ($n = 9$; $P < 0.02$),

whereas the QTc interval remained unchanged (44.1 ± 0.8 ms vs. 43.1 ± 0.5 ms, respectively). The PR intervals were also unchanged between control and CO-exposed rats (45.0 ± 0.5 and 44.0 ± 0.7 ms, respectively). After the animals were challenged with the β -adrenergic receptor agonist isoproterenol (1 mg/kg i.p.), the difference in heart rate between the two groups was abolished, reflecting a weaker response to β -adrenergic stimulation in CO-exposed rats (Figure 1A). The standard deviation of normal-to-normal intervals, an estimate of overall heart rate variability, was decreased in CO-exposed rats (Figure 1A). The number of premature ventricular beats doubled in this group (Figure 1B). The occurrence of nonsustained tachycardia, which is absent in controls, was detected in CO-exposed rats. The number of atrioventricular blocks was unchanged between control and CO-exposed rats (5.2 ± 1.2 and 6.3 ± 1.5 atrioventricular blocks/24 h, respectively). After the animals were

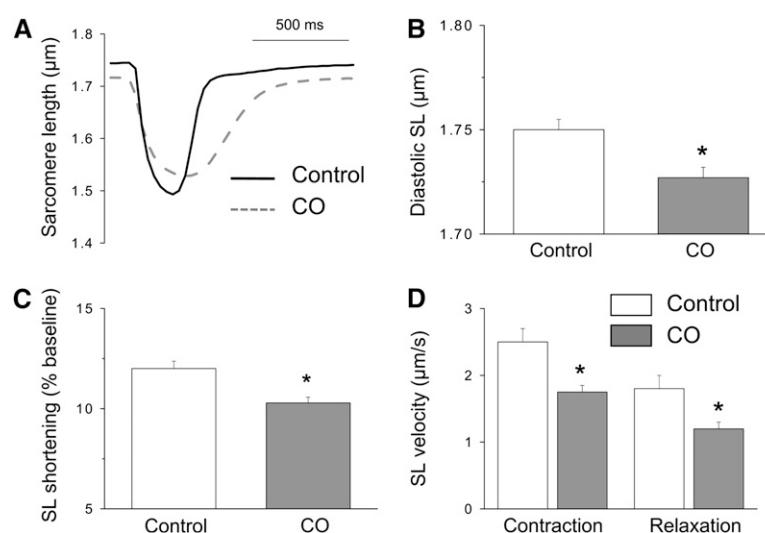
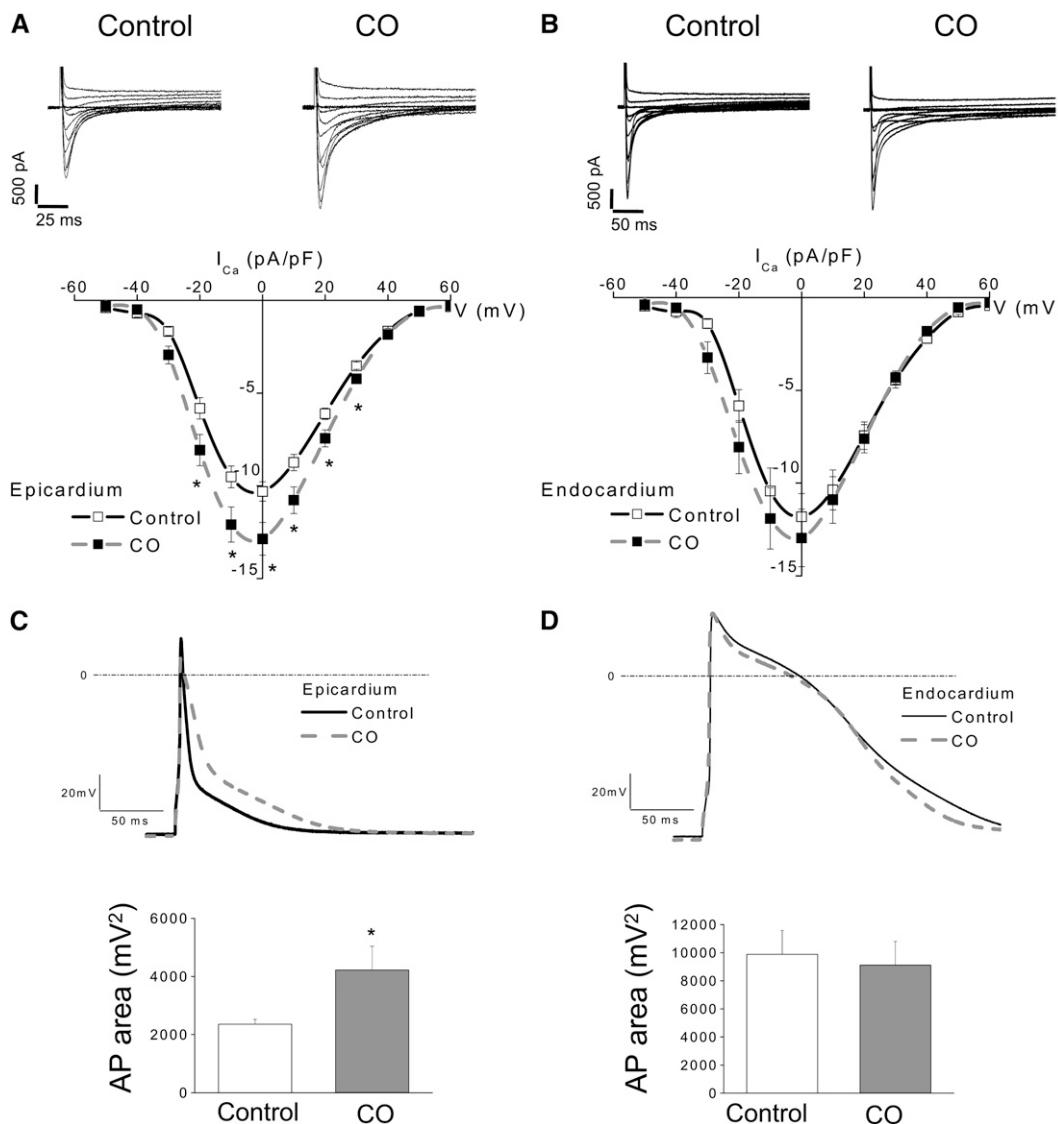


Figure 2. Chronic exposure to carbon monoxide (CO) pollution alters cardiomyocyte contraction. (A) Contraction of intact myocytes, measured by sarcomere length (SL) shortening at 0.5 Hz, isolated from rats exposed to CO pollution (CO, dashed line) and from control rats (solid line). (B) Diastolic SL, (C) the amplitude of SL shortening, and (D) velocities of contraction and relaxation were decreased after chronic CO exposure. Results represent the mean \pm SEM of cells from five hearts ($n = 112-145$). * $P < 0.05$.



challenged with isoproterenol, the number of premature ventricular beats (measured during the 2 h after isoproterenol perfusion) increased in the two groups. However, not only did the absolute risk of arrhythmic events remain greater in the CO-exposed group, but the risk was potentiated (Figure 1B) with the promotion of ventricular ectopic beats that are potential triggers, inducing the start of a sustained arrhythmia.

Effects of CO Exposure on Cardiac ECC

At the cellular level, chronic CO exposure decreased cell fractional shortening (reflecting contraction) in intact ventricular myocytes (Figures 2A and 2C). This decrease was associated with a reduction in diastolic SL (Figure 2B) and in the maximal velocity of cell contraction and relaxation, calculated by the first derivative of cell shortening ($\Delta SL/\Delta$ time) (Figure 1D). These results indicate that ECC is modified by chronic CO exposure.

Because of the well-known heterogeneity in AP duration across the LV free wall (17), we separated subendocardial and subepicardial cells to measure the AP and major ionic currents involved in the AP repolarizing phase. Chronic CO exposure had no effect on cardiomyocyte size, as estimated from measurements of cell membrane capacitance both in epicardial cells

($C_m = 193 \pm 10$ pF, $n = 16$ in controls vs. 203 ± 12 pF, $n = 24$ in the CO-exposed group) and endocardial cells ($C_m = 212 \pm 11$ pF, $n = 13$ in controls vs. 204 ± 13 pF, $n = 20$ in the CO-exposed group), indicating the absence of cellular hypertrophy. In epicardial cells, CO increased the density of L-type inward calcium current ($I_{Ca,L}$) by 20% at 0 mV, and prolonged the AP, as determined from a significant increase in AP area (Figures 3A and 3C). CO had no effect on endocardial cells. In addition, CO had no effect on the repolarizing outward K^+ current in both cell types. Thus, the regional differences in the effects of CO on the AP may have a limited impact on the whole-heart electrical activity.

We next investigated the effects of chronic CO exposure on intracellular Ca^{2+} in Indo-1-loaded cardiomyocytes stimulated at 0.5 Hz. Diastolic (or basal) Ca^{2+} was higher and Ca^{2+} transient amplitude smaller in the CO-exposed group when compared with the control group (Figure 4A). The Ca^{2+} load of the sarcoplasmic reticulum (SR), assessed by caffeine application (10 mM), was also reduced (0.27 ± 0.02 vs. 0.34 ± 0.02 in CO-exposed and control rats, respectively), possibly contributing to Ca^{2+} transient diminution. The increase in diastolic Ca^{2+} could be due to a Ca^{2+} leak through the ryanodine receptors (RyR-2) of the SR during the diastolic phase, or to altered Ca^{2+}

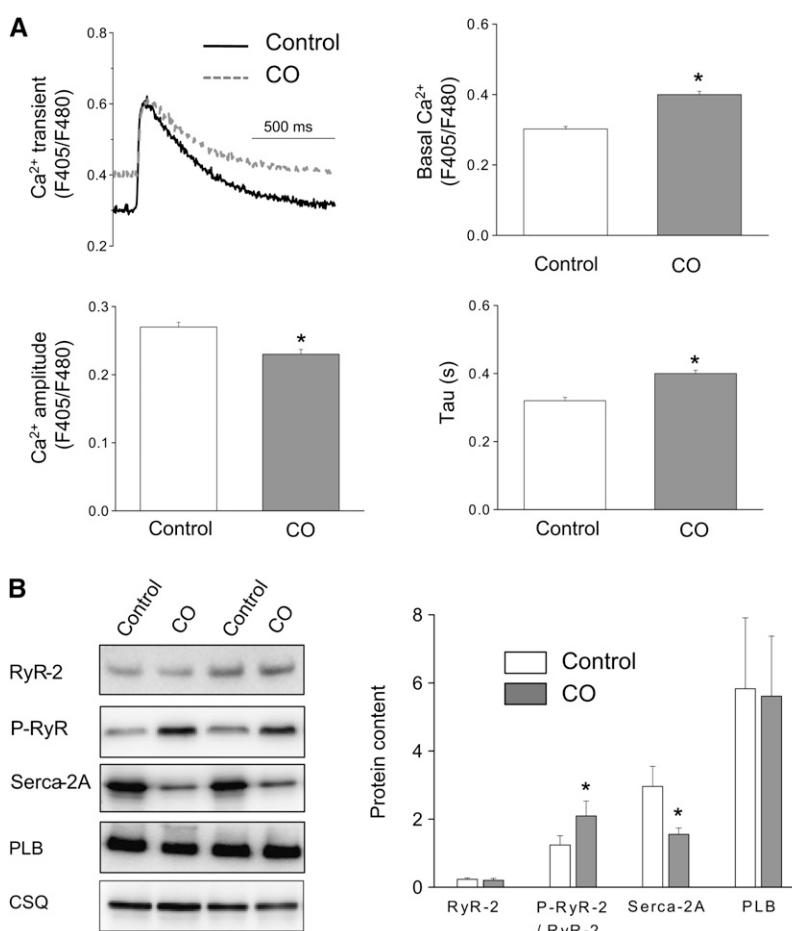


Figure 4. Chronic carbon monoxide (CO) pollution alters the Ca²⁺ dynamics of ventricular myocytes. (A) Superimposed Ca²⁺ transients in intact myocytes isolated from control and CO-exposed rats. Average diastolic intracellular Ca²⁺, Ca²⁺ transient amplitude, and Ca²⁺ reuptake kinetics (τ) ($n = 112\text{--}145$ cells/5 hearts). (B) Effect of chronic exposure to CO on the abundance of Ca²⁺ handling proteins (RyR-2, PLB, and SERCA-2a), analyzed by Western blot analysis. Protein content was normalized to calsequestrin content and Ser²⁸⁰⁹ RyR-phosphorylation normalized to total RyR-2 content. Results are mean \pm SEM for five hearts. * $P < 0.05$.

reuptake by the SR (21). Chronic CO exposure had no effect on RyR-2 protein abundance (Figure 4B). However, it increased the level of RyR-2 phosphorylation on the PKA-dependent site Ser²⁸⁰⁹, which has previously been correlated with a Ca²⁺ leak through the RyR-2 (22). The slowing of the Ca²⁺ transient decay (i.e., increase in τ) in CO myocytes (Figure 4A) is consistent with an impairment of Ca²⁺ reuptake due either to diminished SERCA2a expression or to an inhibition of SERCA2a/PLB activity. We found that chronic CO exposure decreased SERCA-2a expression with no effect on PLB expression (Figure 4B).

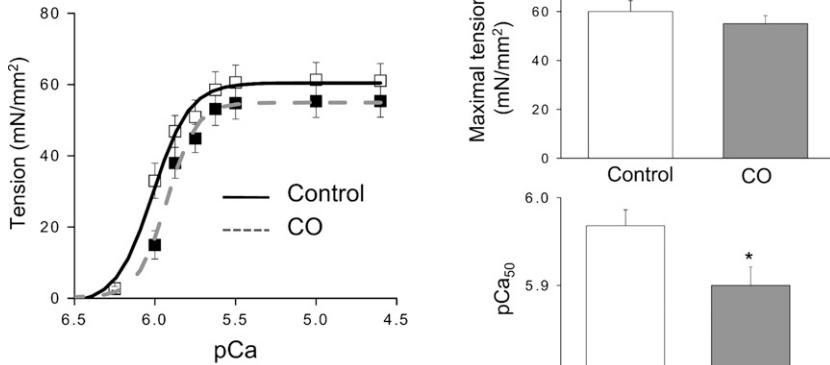
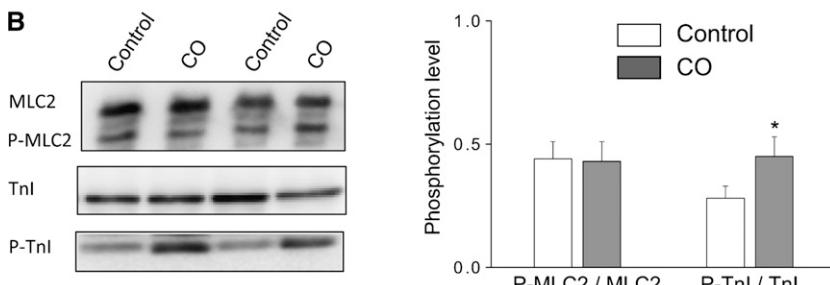
Force developed by cardiomyocytes depends on both the amount of Ca²⁺ released by the SR and Ca²⁺ sensitivity of the contractile machinery. We measured the relationship between Ca²⁺-activated force and internal Ca²⁺ concentration in single permeabilized LV myocytes (Figure 5A). The cross-sectional area in permeabilized cells did not differ between the two groups (203 ± 12 mm² vs. 200 ± 11 mm² in control and CO-exposed rats, respectively), thus confirming the absence of cardiomyocyte hypertrophy revealed by cell capacitance measurements. Maximal isometric tension normalized to cross-sectional area was similar in control and CO-exposed animals. However, a rightward shift in the tension-pCa curve (i.e., decrease in myofilaments Ca²⁺ sensitivity, pCa₅₀) in the CO-exposed group indicated that, for a given amount of Ca²⁺, less force was generated by the myofilaments (Figure 5A). Myofilaments Ca²⁺ sensitivity is regulated by the phosphorylation status of sarcomeric proteins, such as MLC2 and cTnI (23). Chronic CO exposure did not change the phosphorylation level of MLC2 but increased the phosphorylation of cTnI at PKA

sites (Ser^{23/24}) (Figure 5B). Taken together, these data demonstrate that chronic CO exposure alters cardiac cell contractility by modifying various stages of ECC.

Cardiac Response to β -Adrenergic Stimulation

We investigated the effects of CO exposure on the β -adrenergic reserve. *In vivo*, an acute β -adrenergic stimulation (using isoproterenol 1 mg/kg) modified both the maximal rate of increase of LV pressure and the relaxation time constant τ in CO-treated and control rats. However, these effects were less pronounced in the CO group (Figure 6A). Isoproterenol increased the maximal and minimal first derivative of LV pressure by $65.4 \pm 9.1\%$ in controls, but only by $27.9 \pm 9.7\%$ in CO-exposed rats. Similarly, τ decreased by $15.1 \pm 3.3\%$ in controls, but only by $3.7 \pm 3.9\%$ in CO-exposed rats, highlighting a reduction in the lusitropic effect of isoproterenol after chronic CO exposure.

At the cardiomyocyte level, β -adrenergic stimulation activates PKA, which phosphorylates both Ca²⁺-handling proteins involved in ECC (RyR, PLB) and myofilament regulatory proteins (cTnI and MyBP-C). In our study, the positive inotropic effect of isoproterenol (100 nM) on cell shortening was smaller in myocytes from CO-exposed animals than from controls. However, the potentiating effect of isoproterenol on Ca²⁺ transient amplitude was similar in control and CO myocytes (Figure 6B), suggesting that an alteration in the myofilament response is responsible for the decrease in contraction. Permeabilized myocytes were therefore incubated with a recombinant catalytic subunit of PKA (Figure 6C). PKA led to classic myofilament Ca²⁺ desensitization (decrease in pCa₅₀). However this effect was

A**B**

more pronounced in controls than in CO-exposed myocytes, such that the difference in myofilament Ca^{2+} sensitivity disappeared between control and CO myocytes after PKA treatment. Taken together, these data indicate that the β -adrenergic response is altered in animals exposed to chronic CO pollution.

Oxidative Status in CO-exposed Animals

It is well documented that CO binding to cytochrome-c oxidase in the electron transport chain as well as CO interaction with nitric oxide synthase (24) lead to generation of reactive oxygen species (ROS) and nitrogen species (25, 26). We thus evaluated whether the CO-associated cardiac remodeling could involve oxidative stress. Cardiac antioxidant and oxidant statuses are presented in Figure 7. Chronic CO exposure decreased antioxidant enzyme activities of superoxide dismutase, catalase, and glutathione peroxidase. This decrease of antioxidant defenses buffering ROS was associated with increased activity of thioredoxin reductase, confirming a CO-induced oxidative stress (27).

DISCUSSION

It is well established that air pollution is associated with cardiovascular outcome and can cause premature death. However, the mechanisms underlying these effects are poorly understood. We found that exposure of healthy rats to CO for a period limited to 4 weeks, under conditions mimicking urban air pollution, promoted cardiac remodeling and increased arrhythmogenic events. Although only a weak alteration of myocardium function was observed *in vivo*, marked changes in Ca^{2+} handling and Ca^{2+} sensitivity of contractile proteins occurred. To our knowledge, our data provide the first evidence at the cardiomyocyte level that chronic CO exposure promotes a pathological phenotype in the absence of underlying cardiopathy.

A key finding of the present study is that chronic CO exposure depresses the contraction of single rat ventricular myocytes because of reduction of both systolic Ca^{2+} release and myofilament Ca^{2+} sensitivity. In addition, the velocity of both

Figure 5. Effects of carbon monoxide (CO) pollution on contractile machinery in permeabilized cardiomyocytes. (A) The relationship between Ca^{2+} -activated tension and intracellular Ca^{2+} content was measured at 2.3 μm sarcomere length in permeabilized cardiomyocytes isolated from control and CO-exposed rats. The relationship was fitted with a modified Hill equation. The pCa at which half the maximal tension is developed (pCa_{50} , where $\text{pCa} = -\log_{10}[\text{Ca}^{2+}]$) was determined as an index of myofilament Ca^{2+} sensitivity ($n = 25\text{--}28$ cells/5 hearts). (B) Effect of chronic exposure to CO on the phosphorylation level of MLC-2 and cTnI in permeabilized cardiac strips from control and CO-exposed rats. Phosphorylation levels were determined by Western blotting with antibodies to P-MLC2 and to cTnI phosphorylated at the protein kinase A sites Ser²²/Ser²³ (P-cTnI), as described in METHODS. * $P < 0.05$.

the onset and the relaxation of contraction was markedly reduced, in agreement with a slowing of the Ca^{2+} transient decay. The reduced SERCA-2a expression induced by CO exposure is likely to account for the impairment in Ca^{2+} reuptake by the SR, and, therefore, for the delay in cell relaxation. The increase in PKA-phosphorylation of RyR-2 observed in CO-exposed animals is consistent with a Ca^{2+} leak in the SR, known to contribute to intracellular Ca^{2+} overload and SR Ca^{2+} depletion (22, 28, 29). A decrease in SERCA-2a expression could play a role not only in the reduction of SR Ca^{2+} content but also, as a consequence, in the elevation of diastolic Ca^{2+} .

Another major finding of this study is the proarrhythmogenic effect of chronic CO exposure in a healthy population. This result provides experimental support to previous reports that CO is associated with life-threatening VA in populations with cardiopathies (30, 31). The arrhythmic events may have an electrical origin or a Ca^{2+} origin (32). The lack of effect of CO on QT interval, the regionalized effect on AP duration, and the increase in diastolic Ca^{2+} concentration together support the hypothesis that the VAs observed after CO exposure are mediated by Ca^{2+} overload (33). The pathway proposed involves the Ca^{2+} -dependent activation of I_{ti} (the transient depolarizing inward Na^+ current), triggering delayed afterdepolarizations (34), with a facilitating role for β -adrenergic stimulation. Our demonstration of a PKA-dependent hyperphosphorylation of both RyR-2 and TnI is consistent with chronic β -adrenergic overstimulation during CO exposure, and contributes to its proarrhythmogenic effects.

The causative mechanisms involved in the chronic CO pollution-induced pathological cardiac phenotype could have a vascular origin, with limited blood delivery to the cardiac pump (35). Indeed, we demonstrated that, although coronary density was not modified after 4 weeks of CO exposure, perfusion was reduced during a cardiac stress (data not shown). A decreased oxygen delivery due to functional impairment, associated with probable repeated ischemia/reperfusion cycles induced by alternation of CO and ambient air exposure, is very

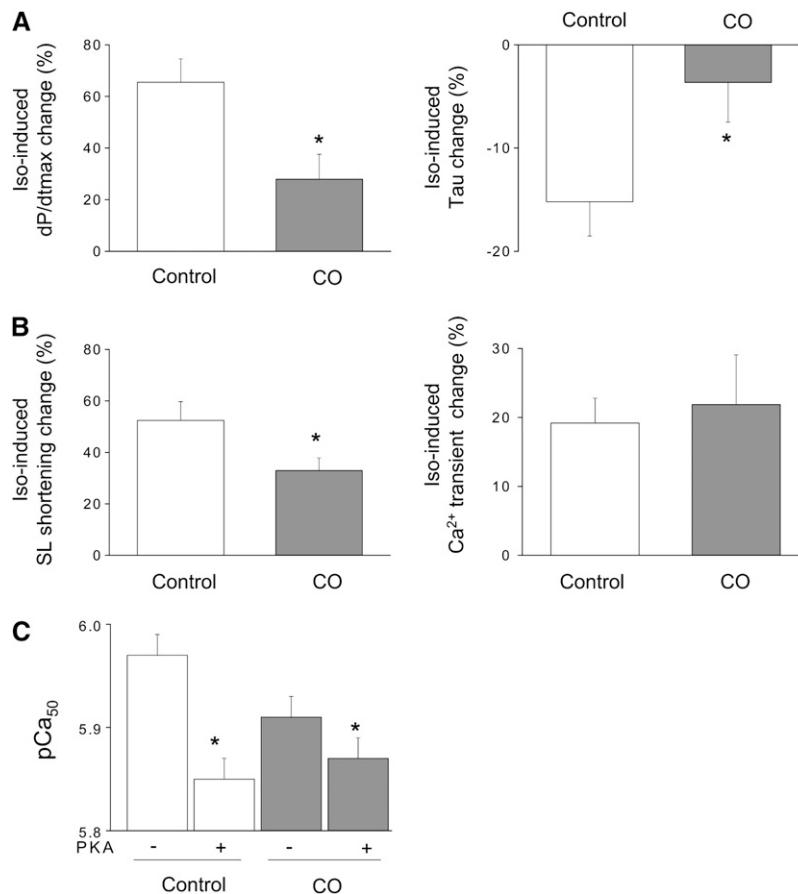


Figure 6. Effect of carbon monoxide (CO) pollution on the β -adrenergic reserve *in vivo* and *in vitro*. (A) Variation of the maximal first derivative (dP/dt max) and relaxation time constant (τ) of left ventricular (LV) pressure after isoproterenol injection (1 mg/kg; iso-induced) evaluated *in vivo* in control and CO-exposed animals ($n = 9$ rats in each group). (B) Variation of contraction (sarcomere length [SL] shortening) and Ca^{2+} transients in isolated LV cells in presence of 100 nM isoproterenol (iso-induced) ($n = 22$ cells for control rats and 37 cells for CO-exposed rats). (C) Myofilament Ca^{2+} sensitivity was similar between control and CO-exposed permeabilized cells after protein kinase A (PKA) treatment (100 U/ml, incubation for 60 min) ($n = 21\text{--}27$ cells/4 hearts). * $P < 0.05$.

likely to induce recurrent stresses and initiate the pathological cardiac remodeling demonstrated in the CO group. Moreover, vascular alterations could enhance the well-established CO-associated production of ROS and nitrogen species (25, 26). Indeed, CO binds to cytochrome-c oxidase of the electron transport chain and interacts with nitric oxide synthase, leading to peroxynitrite generation (24). Previous studies have shown that low-level CO exposure, as for urban pollution, causes oxidative stress (24). In accordance with these studies, we observed a marked increase in thioredoxin reductase activity

in the CO-exposed rats associated with decreased antioxidant enzyme activities, suggesting increased oxidative stress. As proteins involved in Ca^{2+} handling are potential targets for redox modifications (36, 37), the cellular impairments observed in this study could be attributed partially to CO-induced ROS production. We show that chronic CO exposure increases PKA-dependent phosphorylation of both RyR-2 and TnI. ROS may be responsible for the PKA-dependent phosphorylation of cardiac proteins because it has been shown that ROS can activate the PKA pathway (38, 39).

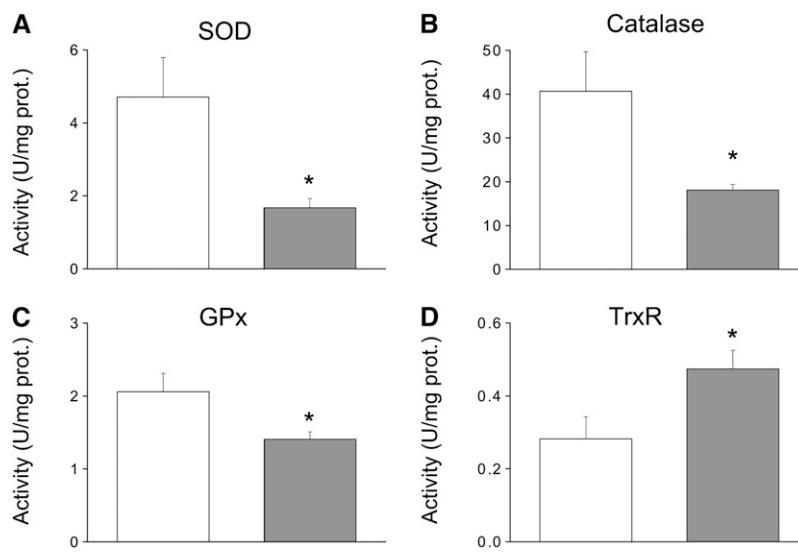


Figure 7. Cardiac oxidative status after chronic carbon monoxide (CO) exposure. Antioxidant activities of (A) superoxide dismutase, (B) catalase, and (C) glutathione peroxidase evaluated in whole heart of control and CO-exposed animals ($n = 7$ rats in each group). (D) Thioredoxin reductase activities, an index of oxidative stress, measured in control and CO-exposed animals ($n = 7$ rats in each group). * $P < 0.05$.

The toxic effect of high concentrations of CO is recognized. However, there is increasing evidence that a low concentration of CO is beneficial in diverse diseases in rodents, large animals, and humans (40). Low CO-dose gas inhalation but also synthetic CO-releasing molecules (CORM) have been recently used as potential therapeutic agents, particularly for cardiovascular diseases (41). CORM reduces infarct size (42), has a protective effect against myocardial infarction (42), prolongs survival of transplanted hearts, increases resistance to hypoxia-reoxygenation (43, 44), and improves cardiac mitochondrial metabolism (45). However, the beneficial cardiac effects of CORM were seen after acute perfusion, which differs from the chronic animal exposure model in the present study. The cardioprotective effects of low CO may be due to inhibition of L-type Ca^{2+} currents (46) occurring via redox modulation of key cysteine residues by mitochondrial ROS (47). In cardiac myocytes, rapid changes in cellular oxygen and ROS generation can contribute to electrophysiological instability and occurrence of arrhythmias via increased L-type Ca^{2+} current (see Reference 48 for review). Although moderate and variable across the LV wall, the increase in L-type Ca^{2+} current reported here might be consistent with an increased oxidative stress. At the moment, CO-based therapeutic strategies use short-term exposure. However, the potential toxicological effect of nontoxic doses of CO (30 ppm/d on average) on cardiac function should be relevant for future investigations of chronic treatment with low-dose CO gas inhalation or CORM.

Study Limitations

The cardiac effects of CO may result from a direct action on the heart and/or from indirect consequences of alterations in the respiratory system function. In fact, the level of carboxyhemoglobin measured 24 hours after the last CO exposure was not different from that of control rats, and was consistent with levels reached in human populations exposed to high atmospheric pollution (12, 13). These levels of carboxyhemoglobin are not reported to be associated, in the literature and in medical practice, with direct hypoxic stress. The pulmonary effects of CO remain controversial and are usually reported after chronic exposure to toxic doses, associated or not with other toxic agents (49, 50). In a set of preliminary experiments, we demonstrated that the mass of the right ventricle was not different between control and CO-exposed rats (0.276 ± 0.015 vs. 0.286 ± 0.015 g, $n = 7$ in each group; $P = 0.64$), whereas that of the LV was significantly increased ($P = 0.007$) after chronic CO exposure (1.02 ± 0.01 vs. 1.11 ± 0.01 g, in control and CO-exposed rats, respectively). Altogether, these data do not support a role for the respiratory system in the cardiac effects of CO reported here.

Clinical Relevance

Chronic CO exposure induced signs of cardiac stress and remodeling with several features commonly observed in pathology, such as HF, wherein impairment of myocardial pump function elicits chronic sympathetic nervous system activity to maintain cardiac output and blood pressure (16). Adrenergic hyperactivation promotes hypertrophy, increases heart rate, favors the development of interstitial fibrosis, and increases the risk of sudden cardiac death (51). Although chronic CO exposure induced only modest alterations in cardiac function *in vivo*, several abnormalities commonly observed in HF were detected. At the integrated level, they included a reduction in fractional shortening and end-systolic strain, parietal hypertrophy of the LV, an increase in fibrosis, an increase in basal heart rate, and a decrease in heart rate variability. At the cellular level, despite the absence of cellular hypertrophy and change in

repolarizing I_{to} density, cardiomyocytes in CO-exposed rats exhibited features typical of HF, including elevated diastolic Ca^{2+} (22), reduced SR Ca^{2+} load (52), decreased systolic Ca^{2+} release (53), a lengthening of Ca^{2+} reuptake (54), and diminished Ca^{2+} sensitivity of the myofilaments (15, 18, 55). In addition, the positive inotropic effect of isoproterenol was partially lost *in vivo* and *in vitro*, consistent with a decrease in the phosphorylation reserve, possibly because of an elevation of the adrenergic (hyperadrenergic) state to compensate for the altered contraction of cardiomyocytes. However, even if the pathological cellular function seems to be rescued *in vivo* under basal conditions, CO-exposed rats exhibited an enhanced arrhythmogenic potential on β -adrenergic stimulation, suggesting an increased risk for cardiac events under conditions of exercise and stress. Interestingly, because ROS are involved in the deleterious effects of CO pollution, antioxidant therapy provided by pharmacology, nutrition, or physical exercise could prevent the alterations reported here.

In conclusion, chronic exposure to CO levels consistent with environmental air pollution promotes moderate cardiac dysfunction *in vivo* in healthy rats. However, serious alterations occur at the cardiomyocyte level, potentially reflecting an early stage of cardiac pathology. Our findings provide a rationale at the cellular and molecular levels in support of earlier epidemiologic studies pointing to the deleterious effects of CO pollution on cardiac function. Further studies will aim at investigating whether the pathologic cellular phenotype evidenced in our study could exacerbate the effects of an acute stress, such as ischemia reperfusion or acute oxidative stress.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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