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Carbon monoxide increases inducible NOS expression which mediates CO-induced myocardial damage during ischemia-reperfusion.

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Abstract

We investigated the role of inducible nitric oxide synthases (iNOS) on ischemic myocardial damage in rats exposed to daily low non-toxic levels of carbon monoxide (CO). CO is a ubiquitous environmental pollutant, which impacts on mortality and morbidity from cardiovascular diseases. We have previously shown that CO exposure aggravates myocardial ischemia-reperfusion (IR) injury partly due to increased oxidative stress. Nevertheless, cellular mechanisms underlying cardiac CO toxicity remain hypothetical. Wistar rats were exposed to simulated urban CO pollution for 4 weeks. Firstly, effects of CO exposure on NO production and NOSs expression were evaluated. Myocardial IR was performed on isolated perfused hearts in the presence or absence of *S*-methyl-isothiourea (SMT, 1µM), a NOS inhibitor highly specific for iNOS. Finally, Ca^{2+} handling was evaluated in isolated myocytes before and after an anoxia-reoxygenation performed with or without SMT or N-acetylcystein (NAC, 20µM), a non specific antioxidant. Our main results revealed that 1) CO exposure altered the pattern of NOS expression, which is characterized by increased nNOS and iNOS expression 2) cardiac NO production increased in CO rats due to its overexpression of iNOS, and 3) the use of a specific inhibitor of iNOS reduced myocardial hypersensitivity to IR (infarct size 29% *vs.* 51% of risk zone) in CO rat hearts. These last results are explained by the deleterious effects of NO and ROS overproduction by iNOS on diastolic Ca^{2+} overload and myofilaments Ca^{2+} sensitivity. In conclusion, this study highlights the involvement of iNOS overexpression in the pathogenesis of simulated urban CO air pollution exposure.

Key words: NO, Myocardial infarction, Carbon monoxide, NOS

1. Introduction

Recently, carbon monoxide (CO) has received a great deal of scientific attention as a biological regulator. Regarding the cardiovascular system, it has been notably reported that endogenous production as well as acute breathing of CO provided cardioprotection (5, 6, 49). However, CO is also a ubiquitous environmental pollutant present in automobile emissions, industrial gases as well as cigarette smoke which impacts on mortality and morbidity from cardiovascular diseases (8). Recently, we and others reported that chronic exposure to sub-toxic low concentrations of CO, relevant to urban environment, induces pathological changes of cardiomyocyte phenotype, with an associated decrease in antioxidant defences, increase in oxidative stress and impairment of Ca^{2+} handling (2, 3, 11, 46, 60). We also pointed out that these alterations rendered the heart more vulnerable to ischemia-reperfusion (IR) (31).

In the heart, nitric oxide synthase (NOS) is well known as key proteins in the modulation of heart sensitivity to IR (15, 51). Both the endothelial NOS (eNOS) and neuronal NOS (nNOS) are low-output Ca^{2+}-dependent enzymes, while the cytokine-inducible NOS (iNOS) is a high-output Ca^{2+}-independent enzyme. eNOS is membrane associated while iNOS and nNOS are soluble and found predominantly in the cytosol. In contrast with constitutive e/nNOS, iNOS is expressed in the myocardium in response to hypoxia, oxidative stress or inflammatory cytokines (59). Up-regulation of iNOS expression results in high levels of nitro-oxidative stress in a variety of cell types and tissues (10). It is well recognized that activation of the eNOS-GMPc-PKG pathway reduces the heart's vulnerability to myocardial infarction (14, 15), whereas the roles of iNOS is still controversial. Indeed, iNOS expression has been shown to be either a key factor in acute strategies of heart preconditioning (25, 53), or to increase myocardial sensitivity to IR (9, 23, 51, 58). In addition, high levels of NO produced

by iNOS are generally associated with pathological cardiac remodeling (34), notably in response to alterations of Ca^{2+} homeostasis (29, 56, 57). In recent literature, nitro-oxidativ stress, resulting mainly from the reaction of high NO levels with superoxide anion ($O_2^{\cdot-}$) to form reactive nitrogen species, has been identified as a major culprit in cardiac IR-injury (28, 33, 37, 51).

Despite it is generally admitted that prolonged CO exposure induces both hypoxic, oxidative (2, 3, 31) and inflammatory stresses in the heart (1), leading to profound rearrangements of the phenotype of myocardial cells, the relationship between chronic exposure to moderate concentrations of CO and the cardiac expression of different isoform of NOS has never been challenged. In the present work, we investigated then, whether: i) CO-induced myocardial stress results in changes in the pattern of NOS expression; and, ii) altered NOS pattern expression in CO-exposed rat heart contributes to pathological effects of CO exposure, subsequently rendering the heart more vulnerable to IR. In our work, CO exposure increased nitrite/nitrate (NOx) level in coronary effluents and iNOS/nNOS expression. Because iNOS rather than nNOS is known as a high generator of NO, and because iNOS expression is well described to aggravate cardiac IR injuries (12, 19), we used a NOS inhibitor (S-methyl-Isothiourea) with higher selectivity for iNOS as compared to nNOS and eNOS (20).

2. Methods

All investigations are conformed to European Parliament Directive 2010/63/EU and have been approved by the local research ethics committee (Comité Régional d'Ethique, experimentation n°: 84.004).

2.1. Animals and carbon monoxide exposure

Adult male Wistar rats (n=66; 367 ± 7 g; Charles River Laboratories) were randomly assigned to 2 groups, carbon monoxide exposed rats (CO rats, exposed for 4 weeks to simulated CO urban pollution, n=33) and Control rats (Ctrl rats, exposed to standard filtered air, n=33). CO rats were exposed during the day for 12 hours to a CO concentration of 30 ppm completed with five 1 hour peaks at 100 ppm as previously described (2, 3, 31). Rats were exposed to CO for 4 weeks and experiments were performed one day after the last exposure to avoid the acute effects of CO on the myocardium. HbCO levels were relatively low at the end of the exposure period with 6.1±1.0% corresponding to an adult working for six to eight hours in air with 50 parts per million (ppm) CO. One day after the last CO exposure, HbCO level was 1.2±0.4%, value similar to HbCO measured in Ctrl rats not exposed to CO (1.1±0.3%).

2.2. Regional myocardial ischemia and reperfusion on isolated perfused rat hearts

After anaesthesia (sodium pentobarbital, 100 mg/kg, i.p.), hearts were in order to perform a regional myocardial IR, as previously described (31). The heart was mounted on a Langendorff isolated heart system and perfused with an oxygenated (95 % O_2 / 5 % CO_2) Krebs solution (37°C) composed of (in mM) NaCl 118.3, $NaHCO_3$ 25, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, Glucose 11.1, CaCl 2.5 (pH= 7.4). Hearts were perfused at a constant pressure (80 mmHg) and were allowed to stabilize for 30 min. Then, a regional ischemia (left anterior descending coronary artery occlusion) was performed during 30 min. Subsequently, the heart was allowed to reperfuse for 120 min. At the end of the protocol, a staining procedure was performed in order to assess infarct size (31). Incidence of ventricular fibrillations (VF) was

evaluated during the first 5 min of reperfusion (31). The IR protocol was performed with or without *S*-methyl-isothiourea (SMT, 1 µM), a potent and highly selective inhibitor for iNOS (190-fold) as compared to eNOS (20), added to the Krebs solution all along the procedure (stabilization, ischemia and reperfusion) (n=8 per condition). We ensured that SMT perfusion has no effect on eNOS, nNOS and iNOS expression by perfusing heart with SMT or not for 1 hour. We found no effect of SMT on eNOS, iNOS and nNOS level in LV.

2.3. Cardiomyocyte excitation-contraction analysis and cellular anoxia/reoxygenation

Single ventricular cardiomyocytes were isolated by enzymatic digestion (32). Cardiomyocytes were transferred into a Petri dish and placed in an anoxic chamber (O_2 level ~ 2 %) for 60 min, followed by a 60-min reoxygenation phase in ambient air (O_2 ~ 19.4 %). Unloaded cell shortening and Ca^{2+} concentration (Indo-1 dye) were measured using field stimulation (0.5 Hz, 22°C, 1.8 mM external Ca^{2+}) before and after anoxia/reoxygenation (A/R). Sarcomere length (SL) and fluorescences (F_{405} and F_{480} nm) were simultaneously recorded (IonOptix system, Hilton, USA). Furthermore, an indirect index of myofilament Ca^{2+} sensitivity was assessed in intact contracting myocytes by calculating the slope of the relationship between shortening and F405:F480 during late relaxation, when it is thought that $[Ca^{2+}]i$ and Ca^{2+} binding to the myofilaments are in equilibrium (43, 47). The experiments were carried out in presence or not of SMT (1 µM) or in presence or not of a large spectrum antioxidant N-Acetylcystein (NAC, 20 mM).

2.4. Biochemical assays

2.4.1. Measurement of ROS generation.

ROS production was measured by electron paramagnetic resonance (EPR) in fresh frozen LV homogenates as previously described (36). Briefly, homogenates were treated with 1 mM 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethyl-pyrrolidine (CMH) solution (1:1 v/v), put in the EPR glass capillary tube (Noxygen Science Transfer & Diagnostics, Germany), and were placed inside the e-scan spectrometer (Bruker, Germany) for data acquisition. The procedure was then repeated on the same samples but in presence of SMT (1µM). ROS production was normalized to the protein content of each sample and then expressed in $\mu mol.min^{-1}.mg^{-1}$.

2.4.2. Lactate dehydrogenase in coronary effluents

Lactate dehydrogenase (LDH) activity was measured in coronary effluents from isolated hearts, by spectrophotometry using an LDH kit (LDH-P, BIOLABO SA, France). Measurements were made at 5, 10 and 15 min of reperfusion. LDH activity was normalized to coronary blood flow and expressed in U/min.

2.4.3. Nitrites/Nitrates in coronary effluents

The amounts of Nitrites/nitrates (NOx) contained in coronary effluents of isolated hearts were used as an index of total NO production and were determined using a quantitative colorimetric assay kit based on the Griess method (QuantiChrom™ Nitric oxide Assay Kit DINO-250). Measurements were made at the end of stabilisation and at 5, 10 and 15 min of reperfusion. NOx concentration was normalized to coronary blood flow and expressed in µmol/min.

2.4.4. Western blot

TNF-α, eNOS, eNOS-p (Ser 1177), nNOS and iNOS expressions and nitrotyrosine protein in LV tissues were evaluated using Western immunoblotting. Proteins were separated

using electrophoresis on a polyacrylamide gel and transferred onto a PVDF membrane. Nonspecific binding sites were blocked with 10 % skimmed milk in Tris-buffered saline solution with 0.5 % Tween for 1 hour at room temperature. Membranes were then incubated overnight at 4°C with primary antibodies directed against eNOS (1/2500, BD transduction), eNOS-p (1/1000, BD transduction), iNOS (1/200, Santa-Cruz Biotechnology), nNOS (1/500, BD transduction), TNF- α (1/1000, Santa-Cruz Biotechnology), nitrotyrosine (1/20000; Millipore Corporation), GAPDH (1/3000, Santa-Cruz Biotechnology) and Tubulin (1/1000, Cell Signaling). Finally, membranes were incubated with corresponding secondary antibodies for 1 hour at room temperature and signals were revealed by chemiluminescence. Optical density of bands was quantified densitometrically after scanning and protein contents were expressed relative to GAPDH.

2.4.5. Measurement of lipid peroxidation

Malondialdehyde (MDA), an end-product of lipid peroxidation, was measured by dot blot analysis as previously described (17). Briefly, homogenized tissues were spotted onto nitrocellulose membranes and incubated with the anti-rabbit MDA (1:500; Millipore Corporation) antibody at 4°C overnight. Signal was revealed as described for western blotting. MDA content was expressed relative to Ponceau S staining.

2.5. Statistics

Data were analyzed using one-way or two-way ANOVA between groups and repeated measures ANOVA when necessary. When significant interactions were found, a Tukey-Kramer test was applied (Statview; SAS Institute, NC, USA). Binomially 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 mean ± Standard error (S.E.).

3. Results

3.1. CO increased inducible NOS expression

Daily exposure of sub-lethal and moderate CO levels relevant with urban levels, stresses the organism of healthy animals that exhibited signs of oxidative stress (2, 3) and inflammation. This is illustrated by the increase of TNF-α expression, a pro-inflammatory cytokine in CO rat hearts compared to Ctrl ones (TNF-α/GAPDH in Ctrl rats: 0.76±0.12, in CO rats: 1.28±0.21; $p<0.05$). Combination of inflammation and oxidative stresses is described in many pathologies and is generally associated with increased expression of iNOS in the cardiovascular system that we explored in the present model. CO exposure increased iNOS expression (Figure 1A), and this higher expression observed using Western blot analysis of whole hearts, was further confirmed by immunostaining of LV myocardial sections with iNOS antibody that showed in CO hearts only clear labelling of cardiomyocytes and a lack of staining of coronary arteries (Figure 1B). The level of expression of eNOS as well as its phosphorylation on its activation site (Serine 1177) were unchanged (Figure 1C). Finally, CO exposure increased the level of nNOS expression (Figure 1D). Increased iNOS and nNOS was associated with a higher myocardial NOx level in coronary effluents of isolated Langendorff hearts from CO rats compared to control rats (Figure 1E), suggesting then higher NO synthesis in the whole heart. In presence of SMT (1 µM), used to inhibit specifically iNOS, the level of NOx produced in CO rat hearts was normalized. Meantime SMT incubation had no effect on NOx level in control rats. The results suggest that the

higher production of NO induced by CO was mainly related to iNOS signalling pathway. We thus focused next on the involvement of iNOS in the deleterious effects of chronic CO.

3.2. Impact of increased iNOS expression on EC coupling of intact cardiomycoytes.

We next evaluated the impact of increased iNOS expression on cellular contractility of intact isolated cardiomyocytes isolated from control and CO-exposed rats in basal conditions. Cell fractional shortening was reduced in CO-exposed myocytes compared to control myocytes (Figure 2A). The lower shortening in CO-exposed myocytes was associated with altered calcium homeostasis as indicated by the reduced Ca^{2+} transient amplitude (Figure 2B, left panel) and the increased diastolic Ca^{2+} level (Figure 2B, right panel). Incubation of CO-exposed myocytes with the iNOS inhibitor SMT restored cell fractional shortening (Figure 2A) but had only a moderate effects on Ca^{2+} handling in CO myocytes (Figure 2B, right panel). This result indicated that the restoration of cell shortening after SMT treatment may be also due to an effect on another component of EC coupling, such as the contractile machinery. We measured the slope of the cell shortening – $[Ca^{2+}]_i$ loop during the late stage of relaxation which has been considered as a reflect of myofilament Ca^{2+} sensitivity in intact myocytes (Figure 2C). The slope was significantly decreased in CO exposed rats compared with control rats, suggesting decreased myofilament Ca^{2+} sensitivity. SMT treatment of CO myocytes only partially restored the index of myofilament Ca^{2+} sensitivity, implying that part of the beneficial effects of iNOS inhibition on cell shortening were mediated through an effect on the contractile machinery. Altogether, these results highlighted the notion that chronic CO exposure increases the production of NO via iNOS in basal conditions that subsequently alters myocyte shortening and could constitute a defavorable substrate for future cardiac stress such as ischemic events.

245 3.3. Involvement of iNOS in myocardial ischemia-reperfusion injuries

246 Though iNOS is known to produce high level of NO in pro-oxidant conditions such as
247 ischemia-reperfusion, iNOS could be uncoupled and then produce $O_2^{\cdot -}$ instead of NO (35). We
248 measured then reactive oxygen species production by electron paramagnetic resonance and
249 NOx level in CO and Ctrl hearts during early post-ischemic reperfusion (ischemia 30 min;
250 reperfusion 10 min). While, no difference of ROS production was observed between the two
251 groups in basal conditions (Ctrl: 119.9±11.7 vs CO: 120.6±9.6 $\mu M.min^{-1}.mg^{-1}$ of LV), during
252 early reperfusion, total ROS production was increased in CO exposed hearts when compared
253 to Ctrl ones. Interestingly, blockade of iNOS by SMT normalized ROS production in CO rat
254 hearts to control value (Figure 3A, right panel). These results were confirmed by MDA
255 content in heart after 10 minutes of reperfusion. Indeed, MDA content was increased in CO
256 rats hearts compared to Ctrl ones, and normalized in presence of SMT (Figure 3B). The same
257 phenomenon was observed with NOx level during early reperfusion. Indeed, in hearts from
258 CO-exposed rats, the amount of NOx released remained higher after ischemia-reperfusion
259 when compared to Ctrl (Figure 3B). Pretreatment of the hearts with the iNOS inhibitor SMT
260 normalized the level of NOx in CO-exposed rat myocardium to the levels of control rats
261 (Figure 3B). SMT incubation had no effect on NOx level in control rats (Figure 3B). Since,
262 NO and $O_2^{\cdot -}$ can react to produce peroxynitrite (ONOO-), we measured by western blot the
263 level of nitrotyrosine after 10 minutes of reperfusion. Despite nitrotyrosine level tended to be
264 increased in CO hearts and to be reduced by SMT, which was not the case in Ctrl ones (Figure
265 3D), any statistical difference was reported between groups regarding this parameter. The
266 involvement of iNOS in the CO-induced cardiac vulnerability was next tested in
267 cardiomyocytes exposed to a protocol of anoxia-reoxygenation (A/R). After A/R, SL
268 shortening was more reduced in cardiomyocytes of CO-exposed rat hearts compared to
269 controls (Figure 4A left panel and 4B). In CO rats, this was associated with reduced Ca^{2+}

transient amplitude (Figure 4A right panel and 4C) and increased diastolic Ca^{2+} overload (Figure 4A right panel and 4D) when compared to control values. In addition, after A/R, myofilament Ca^{2+} sensitivity index obtained in intact cardiomyocytes was markedly reduced in CO rats compared to controls (Figure 4E). In the presence of SMT, SL shortening in cardiomyocytes of CO rats reached the same level as control cells (Figure 4B). The beneficial effect of SMT in CO-exposed myocytes was also observed on Ca^{2+} transient (Figure 4C), diastolic Ca^{2+} levels (Figure 4D), and myofilament Ca^{2+} sensitivity index (Figure 4E). The involvement of oxidative stress in the defects observed when tested by performing A/R in presence of the antioxidant NAC. Pre-incubation of CO-exposed myocytes with NAC prior to anoxia had similar beneficial effects on cell shortening and Ca^{2+} homeostasis as SMT. However, there was no significant effect of NAC treatment on myofilament Ca^{2+} sensitivity index in CO-exposed myocytes (Figure 4E). Altogether the results suggest that chronic CO exposure increases the deleterious effects of A/R via both nitrosative and oxidative pathways. The inhibition of iNOS or the use of an antioxidant protected the myocytes against the CO-induced alterations, in particular the cytosolic Ca^{2+} overload in CO rat cells (Figure 4D), which is recognized as a main trigger of IR injuries (45).

Next, considering that the specific inhibition of iNOS blocked the deleterious effect of CO in cardiomyocytes following anoxia-reoxygenation, we evaluated the involvement of increased iNOS expression in the whole heart vulnerability to regional IR of CO-exposed rats. The potential involvement of iNOS in the higher sensitivity to IR induced by CO observed in cardiomyocytes was also tested in whole heart. The hearts from CO-exposed rats were more vulnerable to IR as indexed by the cellular damages parameters such as the infarct size and the level of lactate dehydrogenase (LDH) released in coronary effluents (Figure 5A and 5B). LDH is highly expressed in cardiac muscle and its release during tissue damage makes its measurement a good marker of common injuries and disease. The size of infarct zone almost

doubled after IR in CO-exposed rats compared with control rats. Similarly, LDH content in coronary effluents was 3 times higher in CO-exposed rats 5 min after reperfusion compared with control rats, decreased at 10 and 15 minutes but remained higher than control rats (Figure 5B). Inhibition of iNOS with SMT before IR did not avoid ischemic damages but prevented the CO-induced increases in infarct zone and in the level of LDH compared to control rat. Blockade of iNOS had no effect in control animals. The deleterious effect of CO was also obvious regarding myocardial perfusion. Indeed, recovery of myocardial perfusion was lower at 1, 30 and 60 min of reperfusion in CO hearts compared to Ctrl ones (Table 1). Interestingly, though SMT has no effect on this parameter in Ctrl hearts, it normalized recovery of myocardial perfusion in CO hearts at level observed in Ctrl ones all along the reperfusion procedure (Table 1). The higher sensitivity of CO-exposed rat myocardium to IR was also detected electrophysiologically from the occurrence of ventricular fibrillations (VF) at the onset of reperfusion in perfused heart (Figure 5C). Following reperfusion 60 % of CO-exposed rats demonstrated VF compared with 20 % in control rats. Blockade of iNOS had no effect in control rats and normalized the occurrence of VF in CO-exposed rats to levels in control rats. In summary, these results indicated that in healthy animals exposed to standard filtered air, iNOS is not involved in the deleterious effects of IR. However chronic exposure to CO specifically enhanced the level of iNOS expression, that generate a specific deleterious signaling pathway during IR based on overproduction of NO and ROS.

4. Discussion

In the present study, we revealed the implication of iNOS in the higher sensitivity of myocardium to ischemic events after prolonged daily exposure to CO at levels relevant to urban environment such as second hand smoking or urban pollution. The major result is that

iNOS mediates this deleterious effect of chronic CO exposure via a NO/ROS dependent pathway which can be avoided with a specific blocker.

Our study provides evidence that chronic CO exposure upregulates iNOS and nNOS expression in the heart, which results in increased NO production that can be prevented by acute treatment with a selective iNOS inhibitor. Although nNOS overexpression seems to represent a general response to cellular stress (19), the link between CO exposure and upregulation of nNOS, is not clear. Regarding iNOS, it is well-established that iNOS is expressed in response to inflammatory and/or oxidative stresses (48). Thus the increased of both oxidative (2, 4) and inflammatory stresses (increased level of TNFα) reported in our model may trigger the production of iNOS. In addition, increased NO production is in line with the fact that iNOS, rather than the two constitutive isoforms of NOS (eNOS and nNOS), is responsible for generating high levels of NO. Indeed, as opposed to the Ca^{2+}-dependent regulation of constitutive eNOS and nNOS enzymes, iNOS has been described as Ca^{2+} insensitive, likely due to its tight non-covalent interaction with calmodulin and Ca^{2+}, and can therefore produce large amounts of NO up to 100 fold greater than normal levels in cardiac cells (13, 34, 55). Though the role of nNOS in increased heart sensitivity to IR was not studied, which constitute a limit of this work, whether nNOS play a role during IR is today not clear (7). Further study will be needed to better understand the complex role of nNOS in CO-induced increased vulnerability to IR.

We have previously shown that prolonged exposure to CO was associated with alterations of cardiomyocyte EC coupling (2) characterized by reduced Ca^{2+} transient amplitude and Ca^{2+} sensitivity of myofilaments (2). It is now well established that NO release can directly influence contractile function of cardiac cells (42), and that a high NO

concentration has negative inotropic effects (39). In the present study, the specific inhibition of iNOS restored the basal contractility of CO isolated cardiomyocytes, with only a slight effect on Ca^{2+} transient amplitude. The main positive effect of iNOS inhibition in our model seems to occur on myofilament function (Figure 2C). Although this result remains to be precisely explored in future experiments it concurs with previous research, showing that high NO levels alter myofilament Ca^{2+} responsiveness by a PKG dependent phosphorylation of tropinin I (TnI) at $Ser^{23/24}$ (27). Such hyperphosphorylation of TnI was previously reported in our model of CO exposure (2). We could thus hypothesize that NO-induced PKG phosphorylation of TnI reduced myofilament Ca^{2+} responsiveness and could participate in the reduction of cardiomyocyte contractility in CO-exposed rats, although this has yet to be confirmed by specific experiments on the contractile machinery.

Prolonged CO exposure induces modest alterations on cardiac function but induces a phenotypical remodelling of the cardiomyocytes that renders the heart more sensitivity to IR (18, 31). The present study indicates that the higher vulnerability of CO rats myocardium to ischemic events is due to increased iNOS expression, which produces high NO levels before and after ischemia- reperfusion but also produces more ROS during early reperfusion. Previously it has been shown that iNOS overexpression protects the heart against IR (53) and constitutes a main trigger of cardiac pre-conditioning (21). However, it also appears that iNOS expression was associated with increased apoptosis during IR (24, 51) and that reduced induction of iNOS reduced heart vulnerability to IR (58). Indeed, in pro-oxidant conditions such as ischemia reperfusion, iNOS overexpression is likely related to uncoupled activity and increased $O_2^{\cdot -}$ instead of NO generation (35). We reported here that increased ROS production and oxidative stress (MDA content) in CO exposed rat hearts during early reperfusion was blunted by the specific inhibition of iNOS with SMT. Consequently, the use of a specific inhibitor of iNOS blunted NO and ROS overproduction during early reperfusion and

normalized heart vulnerability to IR, as evidenced by reduced infarct size and ventricular fibrillation. Some direct antioxidant effect of SMT are doubtful since the use of SMT has no effect on those parameters in Ctrl hearts. The increase in iNOS-dependent ROS production could also suggest an uncoupling of iNOS during IR in CO hearts, however high level of NOx during reperfusion, normalized by SMT, strongly suggest that iNOS was still coupled and able to produce NO. The higher production of ROS during IR in CO hearts could be also explained by the deleterious effects of CO on myocardial enzymatic antioxidant status (31). We cannot also neglect the potential role of the mitochondria during IR, since prolonged CO exposure has been reported to alter mitochondrial function and to increase ROS production during stress (11). Moreover, it has recently been shown, using liver IR model, that iNOS-derived NO could enhanced mitochondrial ROS production in a feed-forward loop that aggravate IR injuries (52). This hypothesis is in addition supported by the fact that incubation of cardiomyocytes with an antioxidant (NAC) has almost the same protective effect than the specific inhibition of iNOS. However, further studies will be needed to understand this phenomenon in CO hearts. In addition, although basal NO production protects cardiomyocytes from death (30, 38), NO overproduction also promotes cellular damages (22, 40). Indeed, NO is a short-lived and relatively unreactive radical, but under pro-oxidative conditions, high amounts of NO can combine with superoxide to form peroynitrite (ONOO-), known to play a significant role in NOS-mediated post-IR cell damages (28, 50, 58). However, in our model, considering that protein nitrotyrosination, used as an index of ONOO- formation only tended to be increased in CO hearts and was only slightly reduced by SMT, it seems doubtful that ONOO- constitute a main trigger of higher heart vulnerability in our model. When produced by eNOS, NO mainly contributes to normal contractile function of heart cells (30, 38), and has even been described as being cardioprotective in IR (14, 15). In our conditions, we found that in normal heart iNOS is not involved in the deleterious effects

of IR. However increased expression of iNOS as observed in some pathological states may produce large amounts of NO/ROS, increase the vulnerability to IR and contribute to pathological remodelling and functional incoherencies of the heart (34, 56, 57). Consequently, the inhibition of iNOS was found to be protective in hearts submitted to IR (23, 54, 58). Oxyradical generation and reactive nitrogen species formation play an important role in the development of intracellular Ca^{2+} overload in cardiomyocytes as a consequence of IR injury (16, 41). Particularly, in pathological conditions, reactive oxygen species is responsible for an alteration of Ca^{2+} handling proteins (29, 44, 57), leading to intracellular Ca^{2+} overload. In myocytes from CO-exposed rat, normalization of NO production by blockade of iNOS or scavenging of excessive ROS production during A/R reduced intracellular Ca^{2+} overload. Moreover Ca^{2+} responsiveness of the myofilaments could also play a key role in altered post-ischemic functional recovery. Accordingly, during IR, depressed myofilament Ca^{2+} responsiveness is a main factor in myocardial stunning (12, 26). Here, iNOS inhibition improved Ca^{2+} sensitivity index in CO-exposed cardiomyocytes after A/R mainly through an inhibition of NO-dependent mechanism, since no significant effect of NAC was observed on myofilament Ca^{2+} sensitivity index after A/R. However, myofilaments Ca^{2+} sensitivity was not measured directly on isolated skinned cardiomyocytes but undirectly using the slope of the relationship between shortening and F405:F480 during late relaxation. They should then be taken very cautiously.

5. Conclusion

In conclusion, we have shown that regular exposure to low CO levels as found during second hand smoking or in polluted urban environment, altered the level of iNOS expression in the myocardium, which renders the heart more sensitive to cardiac stress such as IR.

Specific iNOS inhibition, by reducing NO and ROS overproduction, improves cardiomyocytes function and reduces IR injuries in CO exposed rat hearts.

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7. Disclosures

None

8. Bibliography

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625 **Table 1**

626

	Ctrl	Ctrl SMT	CO	CO SMT
1' ischemia	61,4 ±3,8%	59,9 ± 3,2%	57,6 ± 2,0%	63,2 ± 5,5%
30' ischemia	52,7 ±3,7%	52,8 ± 4,1%	47,2 ± 2,2%	53,0 ± 6,9%
1' reperf.	83,2 ±2,9%	80,1 ± 3,8%	71,3 ± 2,3% *	79,3 ± 3,3% #
30' reperf.	76,8 ±3,0%	81,3 ± 4,2%	65,8 ± 4,1% *	80,4 ± 7,2% #
60' reperf.	65,6 ±1,9%	71,3 ± 5,1%	56,1 ± 3,8% *	74,5 ± 8,1% #

*Table 1: Myocardial perfusion during ischemia reperfusion expressed in % of baseline values ± SEM. * p<0,05 vs.Ctrl; # p< 0,05 vs. CO*

627 **Legends to figures**

628

629 **Fig. 1** Effect of chronic CO exposure on NO signaling. A, Cardiac iNOS expression evaluated
630 by Western immunoblotting and expressed relative to GAPDH content. B, Immunostaining of
631 iNOS expression on control and CO-exposed myocardial slices (red color); CA : coronary
632 artery. C, Cardiac eNOS expression and phosphorylation on its activation site (Ser^{1177})
633 evaluated by Western Immunoblotting and expressed relative to GAPDH and to eNOS
634 content respectively. D, Cardiac nNOS expression evaluated by Western immunoblotting and
635 expressed relative to tubulin content. E, Basal NOx synthesis (Nitrites/Nitrates) measured in
636 coronary effluents of isolated Langendorff hearts with or without specific inhibition of iNOS
637 (SMT). (n=8 per group, ANOVA, **p*<0.05 Ctrl *vs.* CO rats; #p<0.05 SMT *vs.* non-SMT).

638

639 **Fig. 2** Effect of iNOS inhibition on contraction of CO-exposed cardiomyocyte. A, Effect of
640 iNOS specific inhibition on sarcomere length (SL) shortening of CO-exposed
641 cardiomyocytes, B, Ca^{2+} transient amplitude (left panel) and diastolic Ca^{2+} concentration
642 (rigth panel). C, Representative relationship between SL shortening and $[Ca^{2+}]i$ (F405:F480)
643 during cellular contraction and late relaxation (Left panel), effect of CO exposure, with or
644 without iNOS specific inhibition, on myofilament Ca^{2+} sensitivity index (Right panel). (n=4
645 rats/n=20 cells per group, one-way ANOVA, **p*<0.05 Control *vs.* CO rats; # p<0.05 SMT *vs.*
646 without SMT)

647

648 **Fig. 3**.Effect of CO exposure on nitro-oxidative stress during IR. A: Effect of CO exposure
649 and iNOS inhibition with SMT on total ROS production, measured by electron paramagnetic
650 resonance (EPR) in fresh frozen LV homogenates of control and CO exposed rat hearts after
651 10 min of post-ischemic reperfusion. B, Effect of CO exposure and iNOS inhibition with

SMT on MDA content in LV homogenates of control and CO exposed rat hearts after 10 min of post-ischemic reperfusion. C. Effects of CO exposure and iNOS inhibition with SMT, on the production of NOx in coronary effluents of isolated perfused heart after 10 min of post-ischemic reperfusion. D, Effect of CO exposure and iNOS inhibition with SMT on proteins nitrotyrosination in LV homogenates of control and CO exposed rat hearts after 10 min of reperfusion.

Fig. 4 Effect of iNOS inhibition and antioxidant treatment on the sensitivity to anoxia /reoxygenation of cardiomyocytes from control and CO rats. A, Representative contraction (left panel) and intracellular Ca^{2+} signal (right panel) of intact cardiomyocytes following a protocol of anoxia and reoxygenation. B-E, Contraction of intact myocytes in presence or not SMT or NAC was evaluated by measuring sarcomere length (SL) shortening (B), Ca^{2+} transient amplitude (C), diastolic intracellular Ca^{2+} (D), and the myofilament Ca^{2+} sensitivity index (E). Data are expressed relative to Control group and presented as mean ± S.E. (n=4 rats/n=20 cells per group, two-way ANOVA, *p<0.05 *vs.* control rats; # p<0.05 SMT/NAC-treated *vs.* without treatment).

Fig. 5 Effect of iNOS specific inhibition on IR-induced damages of control and CO-exposed rats. A, Representative sections of risk zone (AAR) and infarct size of rat hearts stained respectively with Evans Blue and triphenyltetrazolium chloride (TTC) after 30 min regional ischemia and 120 min reperfusion from isolated heart experiments in each experimental group. Infarct zone expressed as a percentage of risk zone. B, LDH activity measured in coronary effluents at 5, 10 and 15min of reperfusion. (n=8 per group, repeated measures ANOVA, *p<0.05 Ctrl *vs.* CO rats; #p<0.05 SMT *vs.* non-SMT). C, Representative ECG

676 trace of ventricular fibrillation (left panel). Incidence of IR-induced ventricular fibrillation

677 during early reperfusion (right panel)(n=8 per group, Yates' chi square test, $p<0.05$).

678

Figure 1

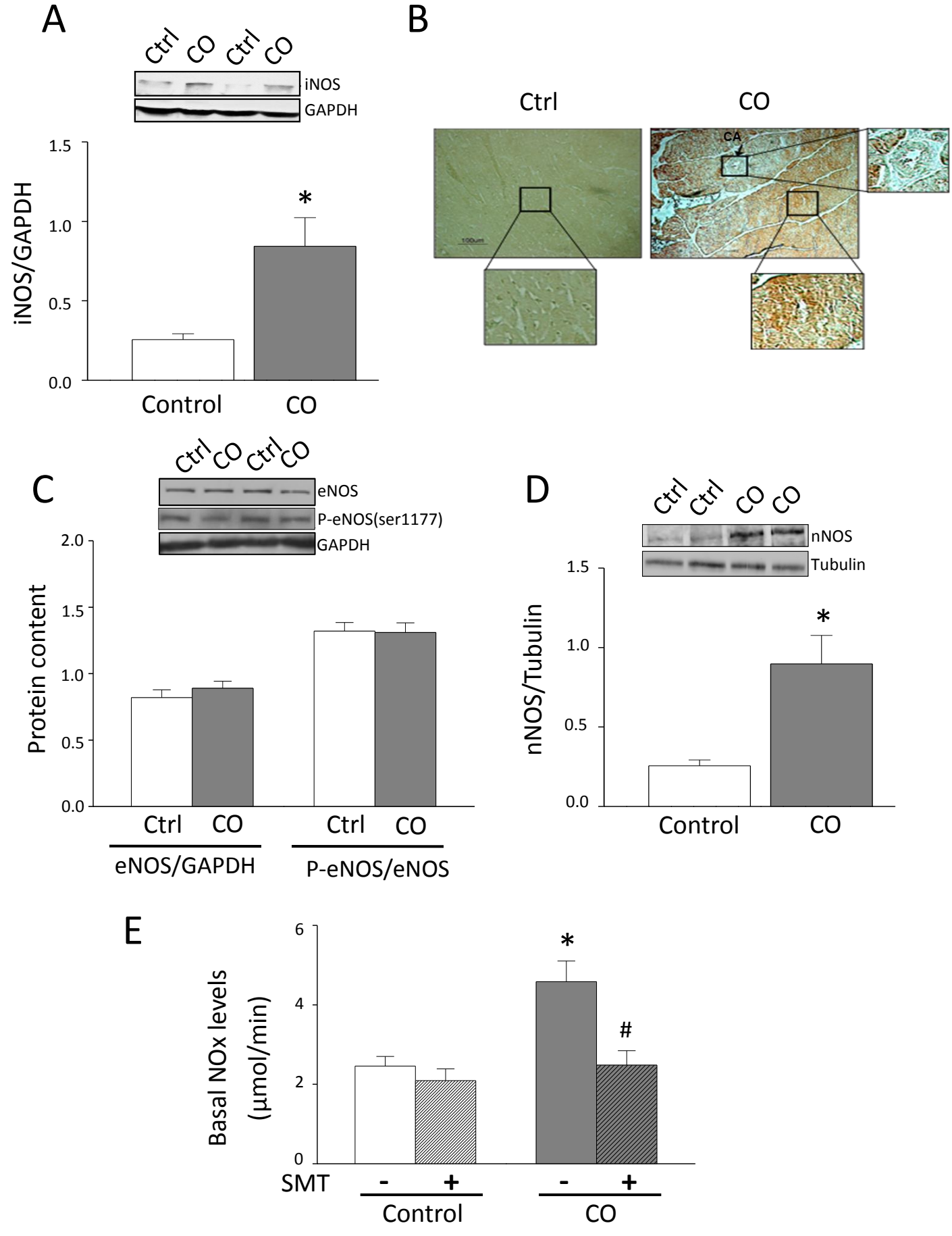

A
Ctrl
CO
Ctrl
CO
iNOS
GAPDH
iNOS/GAPDH
1.5
1.0
0.5
0.0
*
Control
CO
B
Ctrl
CO
CA
C
Ctrl
CO
Ctrl
CO
eNOS
P-eNOS(ser1177)
GAPDH
Protein content
2.0
1.5
1.0
0.5
0.0
Ctrl
CO
Ctrl
CO
eNOS/GAPDH
P-eNOS/eNOS
D
Ctrl
Ctrl
CO
CO
nNOS
Tubulin
nNOS/Tubulin
1.5
1.0
0.5
0.0
*
Control
CO
E
Basal NOx levels
(µmol/min)
6
4
2
0
*
#
SMT
-
+
-
+
Control
CO

Figure 2

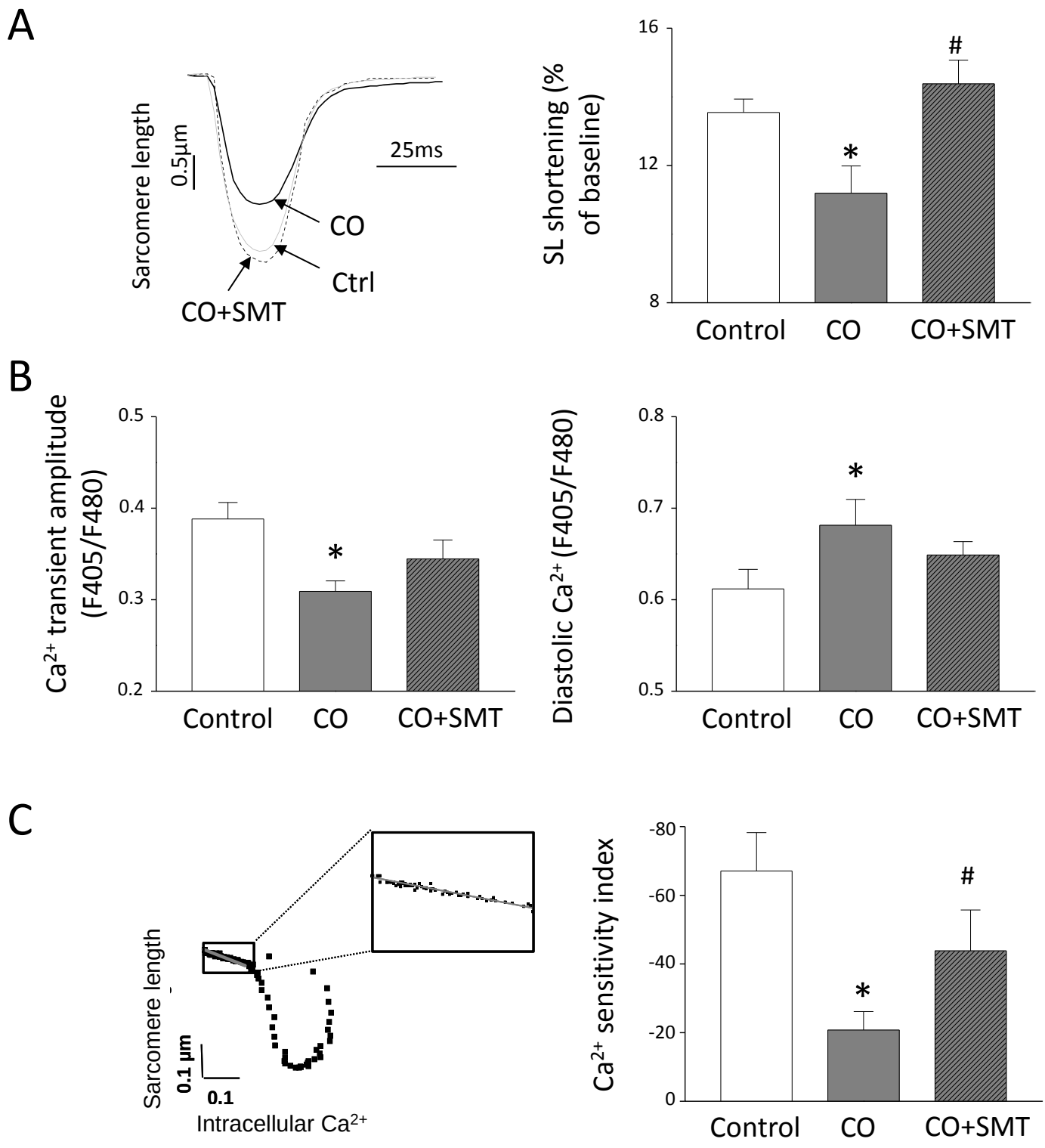

A
Sarcomere length
0.5µm
25ms
CO
Ctrl
CO+SMT
SL shortening (% of baseline)
16
12
8
*
#
Control
CO
CO+SMT
B
Ca2+ transient amplitude (F405/F480)
0.5
0.4
0.3
0.2
*
Control
CO
CO+SMT
Diastolic Ca2+ (F405/F480)
0.8
0.7
0.6
0.5
*
Control
CO
CO+SMT
C
Sarcomere length
0.1 µm
0.1
Intracellular Ca2+
Ca2+ sensitivity index
-80
-60
-40
-20
0
*
#
Control
CO
CO+SMT

Figure 3

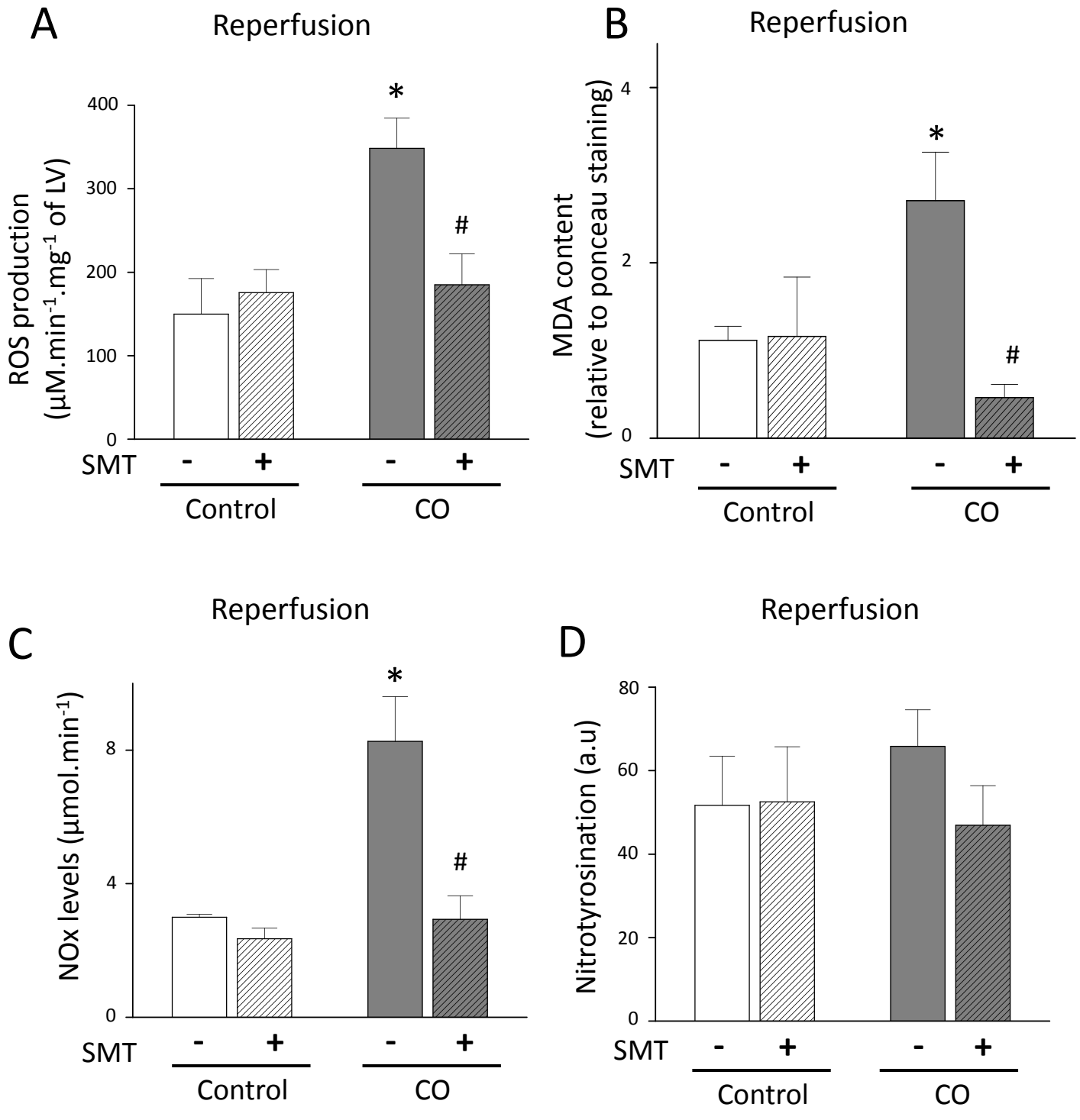

A
Reperfusion
ROS production
(µM.min-1.mg-1 of LV)
400
300
200
100
0
*
#
SMT
-
+
-
+
Control
CO
B
Reperfusion
MDA content
(relative to ponceau staining)
4
2
0
*
#
SMT
-
+
-
+
Control
CO
C
Reperfusion
NOx levels (µmol.min-1)
8
4
0
*
#
SMT
-
+
-
+
Control
CO
D
Reperfusion
Nitrotyrosination (a.u)
80
60
40
20
0
SMT
-
+
-
+
Control
CO

Figure 4

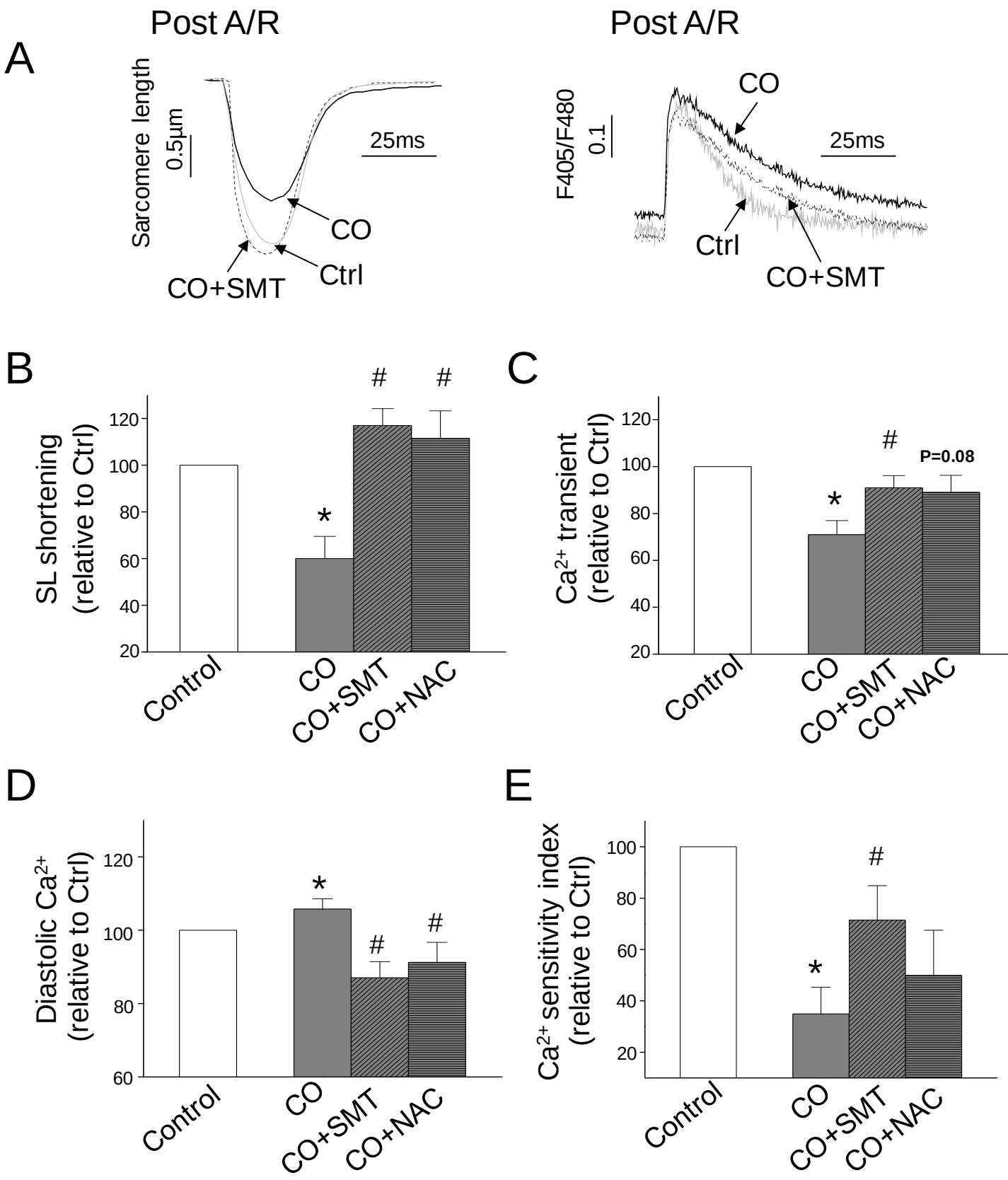
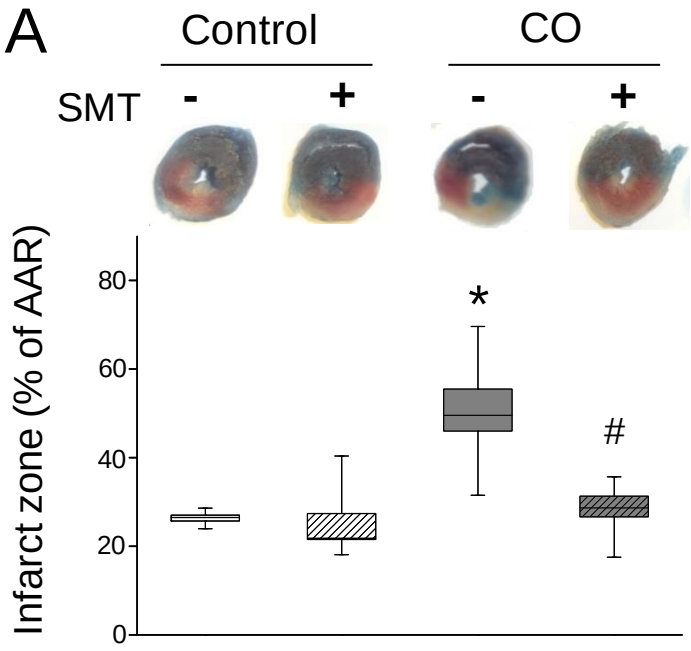
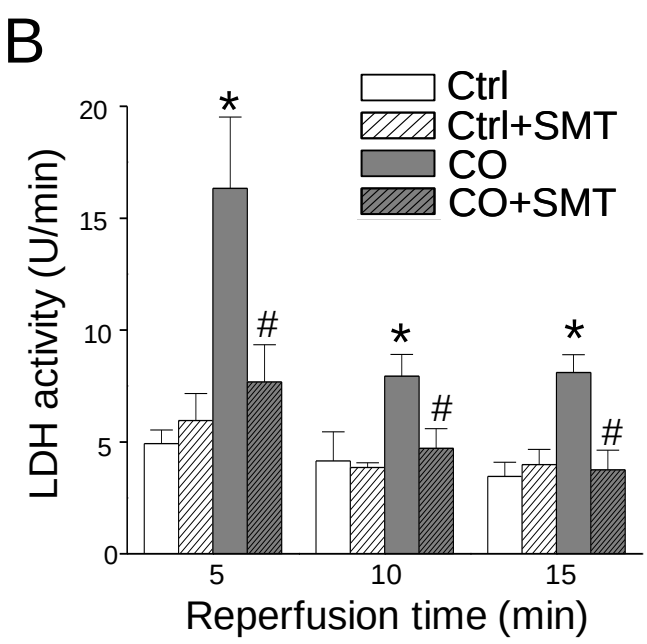
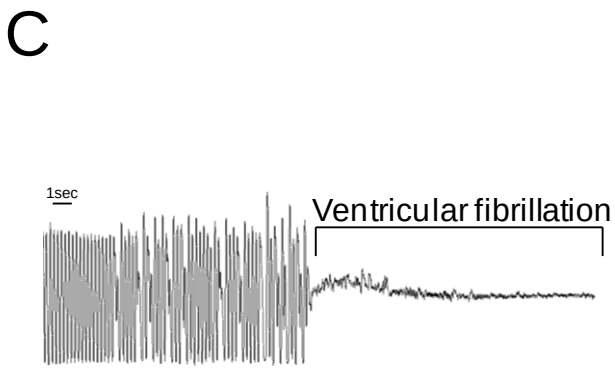
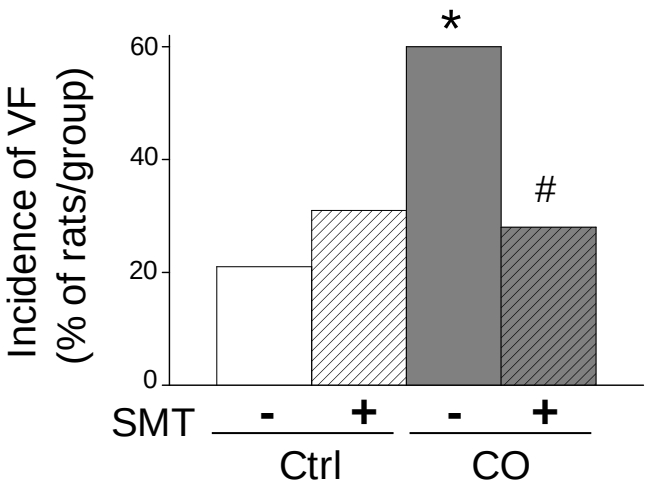

A
Post A/R
Sarcomere length
0.5µm
25ms
CO
Ctrl
CO+SMT
Post A/R
F405/F480
0.1
25ms
CO
Ctrl
CO+SMT
B
SL shortening
(relative to Ctrl)
120
100
80
60
40
20
*
#
#
Control
CO
CO+SMT
CO+NAC
C
Ca2+ transient
(relative to Ctrl)
120
100
80
60
40
20
*
#
P=0.08
Control
CO
CO+SMT
CO+NAC
D
Diastolic Ca2+
(relative to Ctrl)
120
100
80
60
*
#
#
Control
CO
CO+SMT
CO+NAC
E
Ca2+ sensitivity index
(relative to Ctrl)
100
80
60
40
20
*
#
Control
CO
CO+SMT
CO+NAC

Figure 5


A
Control
CO
SMT
-
+
-
+
Infarct zone (% of AAR)
0
20
40
60
80
*
#


B
Ctrl
Ctrl+SMT
CO
CO+SMT
LDH activity (U/min)
0
5
10
15
20
*
#
5
10
15
Reperfusion time (min)


C
1sec
Ventricular fibrillation


Incidence of VF
(% of rats/group)
0
20
40
60
*
#
SMT
-
+
-
+
Ctrl
CO