Aggravation des lésions cardiaques d'ischémie-reperfusion par une pollution de type citadine au CO : Rôle crucial de la iNOS

Carbon monoxide air pollution exacerbates cardiac ischemia-reperfusion injury: a pivotal role for iNOS.

G. Meyer, L. André, S. Tanguy, C. Rugale, S. Gayrard, S. Richard, F. Boucher, O. Cazorla, P. Obert, C. Reboul

II. Etude n°2

1. Résumé article 2

1.1. Contexte scientifique

L'étude n°1 a mis en avant des effets aggravants d'une exposition de type citadine au CO sur la sensibilité du myocarde au syndrome d'IR. Par ailleurs, l'exposition à de faibles concentrations de CO est également à l'origine d'un remodelage pathologique cardiomyocytaire qui semble pouvoir être expliqué par une modification du statut redox cellulaire (Andre et al., 2010). Il est classiquement rapporté dans le cadre de pathologies myocardiques associées à un stress oxydant récurent, une augmentation de l'expression induite d'un isoforme de la NOS, appelé iNOS ou NOS2 (Xie et al., 1994 ; Zhen et al., 2008). Cette enzyme est rapportée dans la littérature scientifique comme étant à l'origine d'une surproduction de NO, notamment expliquée par son absence de régulation post-traductionnelle (Cho et al., 1992 ; Xia, 2007). Par ailleurs, son expression cellulaire est rapportée comme étant responsable, au niveau myocardique, d'un remodelage pathologique (Mungrue et al., 2002 ; Gealekman et al., 2002 ; Massion et Balligand, 2007) associé à une plus grande sensibilité à l'IR (Marfella et al., 2004 ; Hu et al., 2006). Dans notre modèle, nous avons pu observer, dans l'étude n°1, un pic de production de NO au cours des premières minutes de reperfusion (cf. résultats additionnels de l'étude n°1) chez la population de rats CO. En l'absence d'effet du CO sur l'expression de eNOS myocardique (cf. résultats additionnels de l'étude n°1), l'objectif de cette étude n°2 était de répondre au questionnement suivant :

L'exposition au CO, via notamment une modification du statut redox cellulaire, est-elle associée à une expression induite de iNOS cardiaque à l'origine d'une surproduction de NO ? Cette surproduction de NO en condition pro-oxydante peut-elle expliquer la plus grande sensibilité du myocarde au stress d'IR ?

1.2. Méthodologie :

Les conditions expérimentales concernant l'exposition au CO sont similaires à celles décrites dans l'étude n°1. Suite à la période d'exposition, une évaluation du statut redox (activité de la TrxR) et de l'expression tissulaire du TNF-α et de la iNOS est réalisée. Afin d'étudier les effets de l'expression de la iNOS au niveau cellulaire, notamment sur les altérations de l'homéostasie calcique, un protocole d'A/R, en présence ou non de SMT (0,5 µM) (inhibiteur spécifique de iNOS) ou de NAC (20 µM) (antioxydant non spécifique) est réalisé sur cardiomyocytes isolés, chargés avec un indicateur calcique. Enfin, un protocole d'IR régionale sur cœur isolé perfusé de Langendorff est réalisé en présence ou non de SMT pour évaluer l'implication de la iNOS dans la sensibilité accrue à l'IR des myocardes de rats exposés au CO.

1.3. Résultats majeurs :

Un résultat majeur de cette étude est que l'exposition au CO est caractérisée par un état pro-oxydant et un stress inflammatoire à l'origine d'une surexpression de iNOS au niveau myocardique. Ce phénotype a notamment comme conséquence une surproduction de NO dépendante de la iNOS. Enfin, il est intéressant de noter, que l'inhibition spécifique de cette

enzyme au cours du syndrome d'IR, permet, via une inhibition de la surproduction de NO, de normaliser le Ca^{2+} diastolique basale et ainsi la sensibilité du myocarde au syndrome d'IR.

L'exposition au CO est à l'origine d'une surproduction de NO, dépendante d'une surexpression de la iNOS myocardique. Cette production accrue de NO en condition pro-oxydante semble jouer un rôle majeur dans la plus grande vulnérabilité du myocarde au stress d'IR chez les rats exposés au CO.

2. Article n°2 :

Carbon monoxide air pollution exacerbates cardiac ischemia-reperfusion injury: a pivotal role for iNOS.

G. Meyer MSc[1], L. André MSc[2], S. Tanguy PhD[1], C. Rugale MD [3], S. Gayrard BSc[1], S. Richard PhD[2], F. Boucher PhD[4], O. Cazorla PhD[2], P. Obert PhD[1], C. Reboul PhD[1]

[1], Research Laboratory: EA 4278, Physiology and Physiopathology of Cardiovascular Adaptations to Exercise, Faculty of Sciences, Avignon University, F-84000 Avignon, France

[2], Research Laboratory: INSERM U637, Cardiovascular Physiopathology, MONTPELLIER1 University, Faculty of Medicine, F-34295 Montpellier, France

[3], Research Laboratory: EA3127, Groupe Rein et Hypertension, MONTPELLIER1 University, Faculty of Medicine, F-34295 Montpellier, France

[4], Research Laboratory: UMR5525 PRETA-TIMC, Grenoble University Joseph Fourier, Grenoble, France

Running Title: Cardiac toxicity of chronic CO exposure

Contact information: Cyril.reboul@univ-avignon.fr

Phone number: 00.334.90.16.29.32

Fax number: 00.334.90.16.29.01

Abstract

Carbon monoxide (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 in rats. Nevertheless, the cellular mechanisms underlying cardiac CO toxicity still remain hypothetical. The aim of the present study was to investigate whether iNOS expression is involved in the deleterious effects of prolonged CO exposure on myocardial sensitivity to IR. Wistar rats were exposed to simulated urban CO pollution (low level of CO) for 4 weeks. Myocardial IR was performed on isolated perfused hearts in presence or absence of *S*-methyl-isothiourea (SMT, 0.5 µM) a specific iNOS inhibitor. Calcium (Ca^{2+}) handling was evaluated on isolated myocytes after an anoxia-reoxygenation sequence performed with or without SMT or N-acetylcystein (NAC), a non specific antioxidant. Finally, the effects of CO exposure on myocardial NO production and iNOS expression were evaluated. Our main results are that 1) CO exposure induces a pro-inflammatory state to the heart, with an increased cardiac level of TNF-α ; 2) iNOS expression and cardiac NO production are increased in CO rats ; 3) Specific iNOS inhibition reduces CO-induced NO overproduction and reduces infarct size in CO rats (29 % *vs.* 51 % of risk zone). This last result may be explained by the deleterious effects of NO derivatives on Ca^{2+} handling observed at the cardiomyocytes level and probably due to oxidative/nitrative stress. In conclusion, this study highlights the involvement of iNOS over-expression in the pathogenesis of simulated urban CO air pollution exposure.

1. Introduction

Nitric oxide (NO) is produced in eukaryotic cells by a group of three NO synthases (NOS). Two of them are constitutive: the neuronal (nNOS) and the endothelial (eNOS) isoforms; and the third one is inducible (iNOS). Contrarily to constitutive NOS, iNOS expression is upregulated in response to inflammatory cytokines, such as TNF-α, or to oxidative stress (31). Therefore iNOS expression is generally associated with pathological states characterized by pro-inflammatory and/or pro-oxidative background. Because iNOS is irreversibly bound to calmodulin throughout the range of physiological Ca^{2+} concentration, increased iNOS expression is usually associated with elevated NO production (4). Induction of iNOS-expression has been associated with pathological cardiac remodeling (16), notably in response to alterations of calcium (Ca^{2+}) homeostasis (12, 28, 29). Indeed, high concentrations of NO impair Ca^{2+} handling proteins such as the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA-2a) or the ryanodine receptor (RyR-2). In addition, increased iNOS expression has been reported to increase myocardial sensitivity to ischemia and reperfusion (IR) (9, 24, 30). Indeed, in recent literature, oxidative/nitrative stress, resulting mainly from the reaction of NO with superoxide anion ($O_2^{\cdot -}$) to form peroxinitrite ($ONOO^-$), was mainly involved in myocardial damages observed during IR (11, 15, 17, 24). Moreover, in rodent models overexpressing iNOS, specific inhibition of this enzyme markedly reduced myocardial IR injuries (9).

Carbon monoxide (CO) is a ubiquitous environmental pollutant, which was reported to increase cellular oxidative stress (21, 32). Recently, we and others reported that a chronic exposure to low concentration of CO, relevant to urban environment, induces pathological changes of cardiomyocytes phenotypes, with an

associated decrease in antioxidant defences and impairment of Ca^{2+} handling (2, 5). We also highlighted that these alterations render the heart more vulnerable to IR (13).

Today, it is generally recognized that CO exposure induces both oxidative (2, 13, 21, 32) and inflammatory stresses in the heart (1), leading to profound rearrangements of the phenotype of myocardial cells. However, whether low concentrations of CO are responsible for an increased cardiac expression of iNOS has never been challenged. In the present work, we investigated : i) whether CO-induced myocardial stress results in inducible expression of iNOS; and, ii) whether induction of iNOS expression in CO-exposed rat hearts contributes to enhance oxidative/nitrative stress and, thereby, renders the heart more vulnerable to IR.

2. Methods

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

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 housed in an airtight exposure container for 4 weeks. Exposure was performed according to a 12:12-hours CO in air-ambient air cycle, as previously described (2, 13). During CO exposure, a CO concentration of 30 ppm was maintained and completed with five 1 hour peaks at 100 ppm. At the end of the 4 week CO exposure, rats were housed for 24 hours in standard filtered air, in order to avoid the acute effects of CO on the myocardium.

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

On a first set of rats (n=16 per group) a regional myocardial IR was achieved as previously described (13). The IR protocol was performed with or without SMT, a specific iNOS inhibitor at the concentration of 0.5 µM (n=8 per condition). 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). When necessary, SMT was added to the Krebs solution (SMT, 0.5 µM). The hearts were perfused at a constant pressure (80 mmHg) and paced at 300 beats/min with an electrical stimulator (Low voltage stimulator, BSL MP35 SS58L, 3V). The heart was 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 protocol was performed in order to assess infarct size (13).

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

On a second set of rats (n=4 per group), single ventricular cardiomyocytes were isolated by enzymatic digestion (14). Cardiomyocytes were transferred into a glass Petri dish and placed in an anoxic chamber (O_2 level ~ 2 %) for 60 minutes, followed by a 60-minute reoxygenation 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 fluorescence (405 and 480 nm) were simultaneously recorded (IonOptix system, Hilton, USA). The A/R experiment was carried out in presence or not of SMT (0.5 µM) or in presence or not of a non specific antioxidant N-Acetylcystein (NAC, 20 µM).

2.4. Biochemical assays

2.4.1. 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 time. LDH activity was normalized to coronary blood flow.

2.4.2. Nitrites/Nitrates in coronary effluents

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

2.4.3. Immunohistological detection of iNOS expression

On a third set of rats (n=7 per group), LV tissues sections of 5-µm-thick were removed and used for immunohistological study. Identification of iNOS protein was performed using a Rabbit polyclonal anti-iNOS (Santa-cruz). iNOS was quantified using colorimetric quantification (20 X objective).

2.4.4. Thioredoxin reductase activity

On a another set of rats (n=6 per group), hearts 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 w.wt), using a Teflon Potter homogenizer. Tissue homogenates were then centrifuged (10 min at 20.000×g at 4°C). Activities of thioredoxin reductase (TrxR), reflecting the oxidant status, were measured using supernatants as previously described (Andre et al., 2010).

2.4.5. Immunoblotting detection of TNF-α expression

TNF-α expression in LV tissues was evaluated using western immunoblotting. Proteins were separated using 15 % SDS-PAGE. Identification of TNF-α protein was performed using a goat polyclonal anti-TNF-α (Santa-cruz 1:1000) and were expressed relative to GAPDH content.

2.5. Statistics

Data were analyzed using 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). A level of $p<0.05$ was considered statistically significant. Data are expressed as group means or group mean fractions of baseline ± Standard error (S.E.).

3. Results

3.1. CO-induced iNOS expression in myocardial tissues

As assessed by immunostaining, iNOS was overexpressed in CO rats when compared to Ctrl ones (Figure 1A). iNOS expression in tissues can be the consequence of both increased inflammatory cytokines and/or oxidative stress. In our model, both an overexpression of TNF-α (Figure 1B) and an increased oxidative stress, characterized by a higher TrxR activity (Figure 1C) was observed in CO rats compared to controls.

3.2. iNOS induced NO overproduction in coronary effluents

The consequence of the increased iNOS expression was that total NO production, measured in coronary effluents, was significantly higher in hearts from CO rats compared to controls (Figure 1D). The involvement of iNOS in CO induced NO overproduction was confirmed by the incubation of 0.5 µM SMT (a specific inhibitor of iNOS), which prevented this overproduction and maintained its level close to Ctrl level (Figure 1D). No effect of SMT incubation was observed on Ctrl rats.

3.3. Infarct size and myocardial cell death.

Infarct size measurements (Figure 2A) revealed a worsening effect of CO exposure on IR-induced cell death (Figure 2B). The adverse effect of CO exposure on myocardial necrosis was prevented by SMT incubation (Figure 2B). In presence of

SMT, the infarct sizes in CO rats were similar to those of Ctrl animals (Figure 2B). No effect of SMT per se was observed in Ctrl rats (Figure 2AB). Evaluation of LDH release in coronary effluents, used as an index of cell membrane damage, corroborated these results (Figure 2C). Indeed, during post-ischemic reperfusion, LDH release was increased in the CO group when compared to the Ctrl group. This augmentation was prevented by specific inhibition of iNOS with SMT. LDH release was lower in the coronary effluent of CO+SMT rats during reperfusion, and the difference between Ctrl and CO rats was abolished by SMT. No effect of SMT per se was observed on LDH release in Ctrl rats. During IR, NO production evaluated by nitrite-nitrate assessment was increased in CO rats (Figure 2D). This increase was prevented by the specific inhibition of iNOS by SMT incubation, since NO release was significantly lower in the effluents of CO+SMT rats during reperfusion and the difference between Ctrl and CO rats was abolished by SMT (Figure 2D).

3.4. Ca^{2+} handling after anoxia-reoxygenation

At the cellular level, prolonged CO exposure sensitizes ventricular myocytes to A/R (Figure 3). This is characterized by a marked alteration of sarcomere length (SL) shortening in CO rats when compared to Ctrl ones following A/R (Figure 3AB). In addition, higher sensitivity of CO rat cardiomyocytes to A/R was associated with a significant diastolic Ca^{2+} overload (Figure 3CD) resulting in an altered Ca^{2+} transient in this population (Figure 3CE).

To evaluate the potential implication of NO overproduction but also of the reaction of NO with $O_2^{\cdot -}$ to form $ONOO^-$, we have incubated isolated myocytes during the A/R procedure, with a specific inhibitor of iNOS (SMT, 0.5 µM) or with a

scavenger of ROS (NAC, 20 µM). Incubation with NAC reduced the impairment of cardiac cell contraction in both Ctrl and CO rats, confirming the contribution of ROS in the deleterious effects of A/R. In addition, NAC incubation prevented the CO-induced increase in cardiac sensitivity to A/R. Indeed, in this condition, no difference was reported between Ctrl and CO groups regarding SL shortening (Figure 3B). As far as Ca^{2+} handling is concerned, no effect of NAC incubation was observed in both Ctrl and CO rats, therefore the difference between the two experimental populations was still observed (Figure 3E). Finally, the diastolic cytosolic Ca^{2+} overload observed in CO rat cardiomyocytes after A/R was prevented by incubation with NAC and no difference between Ctrl and CO rats was observed (Figure 3D). Specific iNOS inhibition by SMT significantly prevented the increased sensitivity of CO rat cardiomyocytes to A/R (Figure 3). Indeed, the deleterious effects of prolonged exposure to CO on SL shortening and diastolic Ca^{2+} level after A/R were prevented by the specific inhibition of iNOS (Figure 3BD). In presence of SMT, the sensitivity of cardiomyocytes to A/R in CO and Ctrl rats was similar. No effect of SMT incubation was observed during A/R in Ctrl groups (Figure 3).

4. Discussion

To the best of our knowledge, our study is the first to investigate the implication of iNOS-induced NO overproduction in the enhanced sensitivity of the myocardium to IR after prolonged exposure to CO in conditions relevant to urban pollution. The major results are that: i) chronic CO pollution induced expression of iNOS in CO rats myocardium, and ii) specific inhibition of iNOS prevented increased NO production and subsequent worsening of myocardial vulnerability to IR.

4.1. How does CO exposure induce iNOS overexpression?

Our study provides evidence that chronic CO exposure upregulates iNOS expression in the heart, as revealed by immunochemical staining, which resulted in increased NO production. This production, indeed, was prevented specifically by acute treatment with a selective iNOS inhibitor. It is well-established that iNOS is expressed in response to inflammatory and/or oxidative stresses (22, 31). Yet, we show here that inflammatory cytokines and pro-oxidative marker, such as TNF-α and TrxR respectively, were increased in response to prolonged CO exposure. We also previously showed in the same experimental model that CO exposure is associated with lower enzymatic antioxidant activities and altered redox status (2, 13).

The mechanism responsible for upregulation of iNOS in response to oxidative stress is unclear. It is possibly mediated by ROS-induced activation of nuclear factor κB (NFκB) and subsequent initiation of the inflammatory cascade that includes induction of iNOS (10, 31). The close relationship between inflammatory stress and

iNOS expression is in contrast better understood. Indeed, it is well recognized that NFκB -dependent activation of the iNOS promotor promotes an inflammation mediated stimulation of this transcript (27). Then, further studies are needed to illustrate potential interaction between pro-inflammatory/oxidative mediators and iNOS up-regulation in CO exposed rats.

4.2. Implication of iNOS in CO rats' myocardium sensitivity to IR

The major result here was that the higher cardiac vulnerability of 'CO rats' to IR was prevented by specific inhibition of iNOS. As opposed to the Ca^{2+}-dependent regulation of constitutive NOS enzymes, iNOS has been described as Ca^{2+} insensitive, likely due to its tight non-covalent interaction with calmodulin and Ca^{2+} (6, 26). INOS isoform can produce large amounts of NO that are up to 100 times greater than normal level in cardiac cells (16). Basal NO production protects cardiomyocytes from cells death, whereas high production by iNOS promotes cells death (8, 18). Indeed, NO is a short-lived and relatively unreactive radical, but in pro-oxidative condition, high amount of NO can combine with superoxide to form the potent oxidant $ONOO^-$ shown to play a significant role in iNOS-mediated post-IR cell damages (3, 23). It is well recognized that, during IR, interaction between ROS and high amount of NO promotes cell death, notably due to the formation of $ONOO^-$ (11, 30). Consequently, the specific inhibition of iNOS was found to be protective in hearts submitted to IR (9, 11, 25, 30). Such an implication of iNOS-induced NO overproduction in the higher vulnerability of CO rats myocardium to IR was reported in our model. In addition, in our work, specific inhibition of $ONOO^-$ precursors (NO overproduction by iNOS and ROS) blunted the exacerbated sensitivity of CO rat's

myocytes to A/R. This result is notably explained by normalized Ca^{2+} homeostasis alterations. Oxyradical generation and peroxynitrite formation play an important role in the development of intracellular Ca^{2+} overload in cardiomyocytes as a consequence of IR injury (7, 19). Particularly, in pathological conditions, formation of $ONOO^-$ due to iNOS is responsible for an alteration of Ca^{2+} handling proteins through a nitration of SERCA-2a (12), and a s-nitrosylation of RyR (20, 29), leading then to intracellular Ca^{2+} overload. Then, in CO rats' myocardium, the specific inhibition of iNOS, blunting the overproduction of NO, prevents exacerbated IR-induced intracellular Ca^{2+} overload and then normalizes heart vulnerability to IR.

In conclusion, we have shown that urban CO pollution upregulates inducible expression of iNOS, which renders the heart more vulnerable to stress or damages, notably in pro-oxidant conditions such as IR. Specific iNOS inhibition, by reducing NO overproduction, has protective effect and reduce IR injuries in CO exposed rats. A possible explanation of the increased myocardial IR injury consecutive to CO exposure is the deleterious effect of NO overproduction on Ca^{2+} homeostasis, probably mediated by its reaction with ROS to form peroxynitrite.

5. Grants

This work was supported by a French National Research Agency grant (COMYOCARD).

6. Author Disclosure Statement.

No competing financial interests exist

7. Bibliography

1. **Aberg AM, Abrahamsson P, Johansson G, Haney M, Winso O, and Larsson JE.** Does carbon monoxide treatment alter cytokine levels after endotoxin infusion in pigs? A randomized controlled study. *J Inflamm (Lond)* 5: 13, 2008.

2. **Andre L, Boissiere J, Reboul C, Perrier R, Zalvidea S, Meyer G, Thireau J, Tanguy S, Bideaux P, Hayot M, Boucher F, Obert P, Cazorla O, and Richard S.** Carbon monoxide pollution promotes cardiac remodeling and ventricular arrhythmia in healthy rats. *Am J Respir Crit Care Med* 181: 587-595, 2010.

3. **Arstall MA, Sawyer DB, Fukazawa R, and Kelly RA.** Cytokine-mediated apoptosis in cardiac myocytes: the role of inducible nitric oxide synthase induction and peroxynitrite generation. *Circ Res* 85: 829-840, 1999.

4. **Bredt DS.** Endogenous nitric oxide synthesis: biological functions and pathophysiology. *Free Radic Res* 31: 577-596, 1999.

5. **Bye A, Sorhaug S, Ceci M, Hoydal MA, Stolen T, Heinrich G, Tjonna AE, Najjar SM, Nilsen OG, Catalucci D, Grimaldi S, Contu R, Steinshamn S, Condorelli G, Smith GL, Ellingsen O, Waldum H, and Wisloff U.** Carbon monoxide levels experienced by heavy smokers impair aerobic capacity and cardiac contractility and induce pathological hypertrophy. *Inhal Toxicol* 20: 635-646, 2008.

6. **Cho HJ, Xie QW, Calaycay J, Mumford RA, Swiderek KM, Lee TD, and Nathan C.** Calmodulin is a subunit of nitric oxide synthase from macrophages. *J Exp Med* 176: 599-604, 1992.

7. **Dhalla NS, Temsah RM, and Netticadan T.** Role of oxidative stress in cardiovascular diseases. *J Hypertens* 18: 655-673, 2000.

8. **Han W, Fu S, Wei N, Xie B, Li W, Yang S, Li Y, Liang Z, and Huo H.** Nitric oxide overproduction derived from inducible nitric oxide synthase increases cardiomyocyte apoptosis in human atrial fibrillation. *Int J Cardiol* 130: 165-173, 2008.

9. **Hu A, Jiao X, Gao E, Koch WJ, Sharifi-Azad S, Grunwald Z, Ma XL, and Sun JZ.** Chronic beta-adrenergic receptor stimulation induces cardiac apoptosis and aggravates myocardial ischemia/reperfusion injury by provoking inducible nitric-oxide synthase-mediated nitrative stress. *J Pharmacol Exp Ther* 318: 469-475, 2006.

10. **Hur GM, Ryu YS, Yun HY, Jeon BH, Kim YM, Seok JH, and Lee JH.** Hepatic ischemia/reperfusion in rats induces iNOS gene transcription by activation of NF-kappaB. *Biochem Biophys Res Commun* 261: 917-922, 1999.

11. **Li D, Qu Y, Tao L, Liu H, Hu A, Gao F, Sharifi-Azad S, Grunwald Z, Ma XL, and Sun JZ.** Inhibition of iNOS protects the aging heart against beta-adrenergic receptor stimulation-induced cardiac dysfunction and myocardial ischemic injury. *J Surg Res* 131: 64-72, 2006.

12. **Lokuta AJ, Maertz NA, Meethal SV, Potter KT, Kamp TJ, Valdivia HH, and Haworth RA.** Increased nitration of sarcoplasmic reticulum Ca2+-ATPase in human heart failure. *Circulation* 111: 988-995, 2005.

13. **Meyer G, Andre L, Tanguy S, Boissiere J, Farah C, Lopez-Lauri F, Gayrard S, Richard S, Boucher F, Cazorla O, Obert P, and Reboul C.** Simulated urban carbon monoxide air pollution exacerbates rat heart ischemia-reperfusion injury. *Am J Physiol Heart Circ Physiol* 298: H1445-1453, 2010.

14. **Mou YA, Reboul C, Andre L, Lacampagne A, and Cazorla O.** Late exercise training improves non-uniformity of transmural myocardial function in rats with ischaemic heart failure. *Cardiovasc Res* 81: 555-564, 2009.

15. **Mukhopadhyay P, Rajesh M, Batkai S, Kashiwaya Y, Hasko G, Liaudet L, Szabo C, and Pacher P.** Role of superoxide, nitric oxide, and peroxynitrite in doxorubicin-induced cell death in vivo and in vitro. *Am J Physiol Heart Circ Physiol* 296: H1466-1483, 2009.

16. **Mungrue IN, Gros R, You X, Pirani A, Azad A, Csont T, Schulz R, Butany J, Stewart DJ, and Husain M.** Cardiomyocyte overexpression of iNOS in mice results in peroxynitrite generation, heart block, and sudden death. *J Clin Invest* 109: 735-743, 2002.

17. **Pacher P, Beckman JS, and Liaudet L.** Nitric oxide and peroxynitrite in health and disease. *Physiol Rev* 87: 315-424, 2007.

18. **Razavi HM, Hamilton JA, and Feng Q.** Modulation of apoptosis by nitric oxide: implications in myocardial ischemia and heart failure. *Pharmacol Ther* 106: 147-162, 2005.

19. **Saini-Chohan HK and Dhalla NS.** Attenuation of ischemia-reperfusion-induced alterations in intracellular Ca2+ in cardiomyocytes from hearts treated with N-acetylcysteine and N-mercaptopropionylglycine. *Can J Physiol Pharmacol* 87: 1110-1119, 2009.

20. **Stoyanovsky D, Murphy T, Anno PR, Kim YM, and Salama G.** Nitric oxide activates skeletal and cardiac ryanodine receptors. *Cell Calcium* 21: 19-29, 1997.

21. **Thom SR and Ischiropoulos H.** Mechanism of oxidative stress from low levels of carbon monoxide. *Res Rep Health Eff Inst*: 1-19; discussion 21-17, 1997.

22. **Umar S and van der Laarse A.** Nitric oxide and nitric oxide synthase isoforms in the normal, hypertrophic, and failing heart. *Mol Cell Biochem* 333: 191-201.

23. **Walker LM, Walker PD, Imam SZ, Ali SF, and Mayeux PR.** Evidence for peroxynitrite formation in renal ischemia-reperfusion injury: studies with the inducible nitric oxide synthase inhibitor L-N(6)-(1-Iminoethyl)lysine. *J Pharmacol Exp Ther* 295: 417-422, 2000.

24. **Wang XL, Liu HR, Tao L, Liang F, Yan L, Zhao RR, Lopez BL, Christopher TA, and Ma XL.** Role of iNOS-derived reactive nitrogen species and resultant nitrative stress in

leukocytes-induced cardiomyocyte apoptosis after myocardial ischemia/reperfusion. *Apoptosis* 12: 1209-1217, 2007.

25. **Wildhirt SM, Suzuki H, Horstman D, Weismuller S, Dudek RR, Akiyama K, and Reichart B.** Selective modulation of inducible nitric oxide synthase isozyme in myocardial infarction. *Circulation* 96: 1616-1623, 1997.

26. **Xia Y.** Superoxide generation from nitric oxide synthases. *Antioxid Redox Signal* 9: 1773-1778, 2007.

27. **Xie QW, Kashiwabara Y, and Nathan C.** Role of transcription factor NF-kappa B/Rel in induction of nitric oxide synthase. *J Biol Chem* 269: 4705-4708, 1994.

28. **Xu L, Eu JP, Meissner G, and Stamler JS.** Activation of the cardiac calcium release channel (ryanodine receptor) by poly-S-nitrosylation. *Science* 279: 234-237, 1998.

29. **Xu S, Ying J, Jiang B, Guo W, Adachi T, Sharov V, Lazar H, Menzoian J, Knyushko TV, Bigelow D, Schoneich C, and Cohen RA.** Detection of sequence-specific tyrosine nitration of manganese SOD and SERCA in cardiovascular disease and aging. *Am J Physiol Heart Circ Physiol* 290: H2220-2227, 2006.

30. **Zhao X, Chen YR, He G, Zhang A, Druhan LJ, Strauch AR, and Zweier JL.** Endothelial nitric oxide synthase (NOS3) knockout decreases NOS2 induction, limiting hyperoxygenation and conferring protection in the postischemic heart. *Am J Physiol Heart Circ Physiol* 292: H1541-1550, 2007.

31. **Zhen J, Lu H, Wang XQ, Vaziri ND, and Zhou XJ.** Upregulation of endothelial and inducible nitric oxide synthase expression by reactive oxygen species. *Am J Hypertens* 21: 28-34, 2008.

32. **Zuckerbraun BS, Chin BY, Bilban M, d'Avila JC, Rao J, Billiar TR, and Otterbein LE.** Carbon monoxide signals via inhibition of cytochrome c oxidase and generation of mitochondrial reactive oxygen species. *FASEB J* 21: 1099-1106, 2007.

Legends to figures

Fig. 1 A, Effects of CO exposure on iNOS expression evaluated by immunostaining on myocardial slices, quantified by colorimetric assessment (red color) and expressed in arbitrary unit. Data are presented as mean ± S.E. (one-way ANOVA, * p<0.05 *vs.* Ctrl rats). B, Effects of CO exposure on TNF-α expression, expressed relative to GAPDH content. Data are presented as mean ± S.E. (one-way ANOVA, * p<0.05 *vs.* Ctrl rats). C, Effects of CO exposure on thioredoxin reductase (TrxR) activity expressed in U/mg of protein. Data are presented as mean ± S.E. (one-way ANOVA, * p<0.05 *vs.* Ctrl rats). D, Effects of iNOS inhibition and CO exposure on NO synthesis (Nitrites/Nitrates). Data are presented as mean ± S.E. (n=10 per group, repeated measures ANOVA, *p<0.05 Ctrl *vs.* CO rats; #p<0.05 SMT *vs.* non-SMT).

Fig. 2 Effect of iNOS inhibition and CO exposure on IR-induced cellular death. A, Representative sections of 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. B, Infarct zone expressed as a percentage of risk zone. Data are presented as mean ± S.E. (n=8 per group, one way ANOVA, *p<0.05 Ctrl *vs.* CO rats; #p<0.05 SMT *vs.* non-SMT) C, LDH activity observed in coronary effluents at 5, 10 and 15 min of reperfusion and used as a marker of cell death. Data are presented as mean ± S.E. (n=8 per group, repeated measures ANOVA, *p<0.05 Ctrl *vs.* CO rats; #p<0.05 SMT *vs.* non-SMT). D, Effects of iNOS inhibition and CO exposure on NO synthesis at 5, 10 and 15 min of reperfusion time during IR protocol. Data are

presented as mean ± S.E. (n=8 per group, repeated measures ANOVA, *p<0.05 Ctrl *vs.* CO rats; #p<0.05 SMT *vs.* non-SMT).

Fig. 3 Effects of iNOS inhibition and antioxidant treatment on the sensitivity to A/R of cardiomyocytes from Ctrl and CO rats. A, Representative contraction of intact cardiomyocytes measured by sarcomere shortening at 0.5 Hz. B, Contraction of intact myocytes, measured by sarcomere length (SL) shortening. Data are presented as percentage of baseline ± S.E. C, Representative Ca^{2+} transient during cardiomyocyte excitation-contraction. D, Diastolic intracellular Ca^{2+} and E, Ca^{2+} transient amplitude in intact myocytes. Data are presented as mean ± S.E. (n=4 per group, two-way ANOVA, *p<0.05 Ctrl *vs.* CO rats; # p<0.05 SMT *vs.* without SMT; $ p<0.05 NAC *vs.* without NAC).

Fig. 1

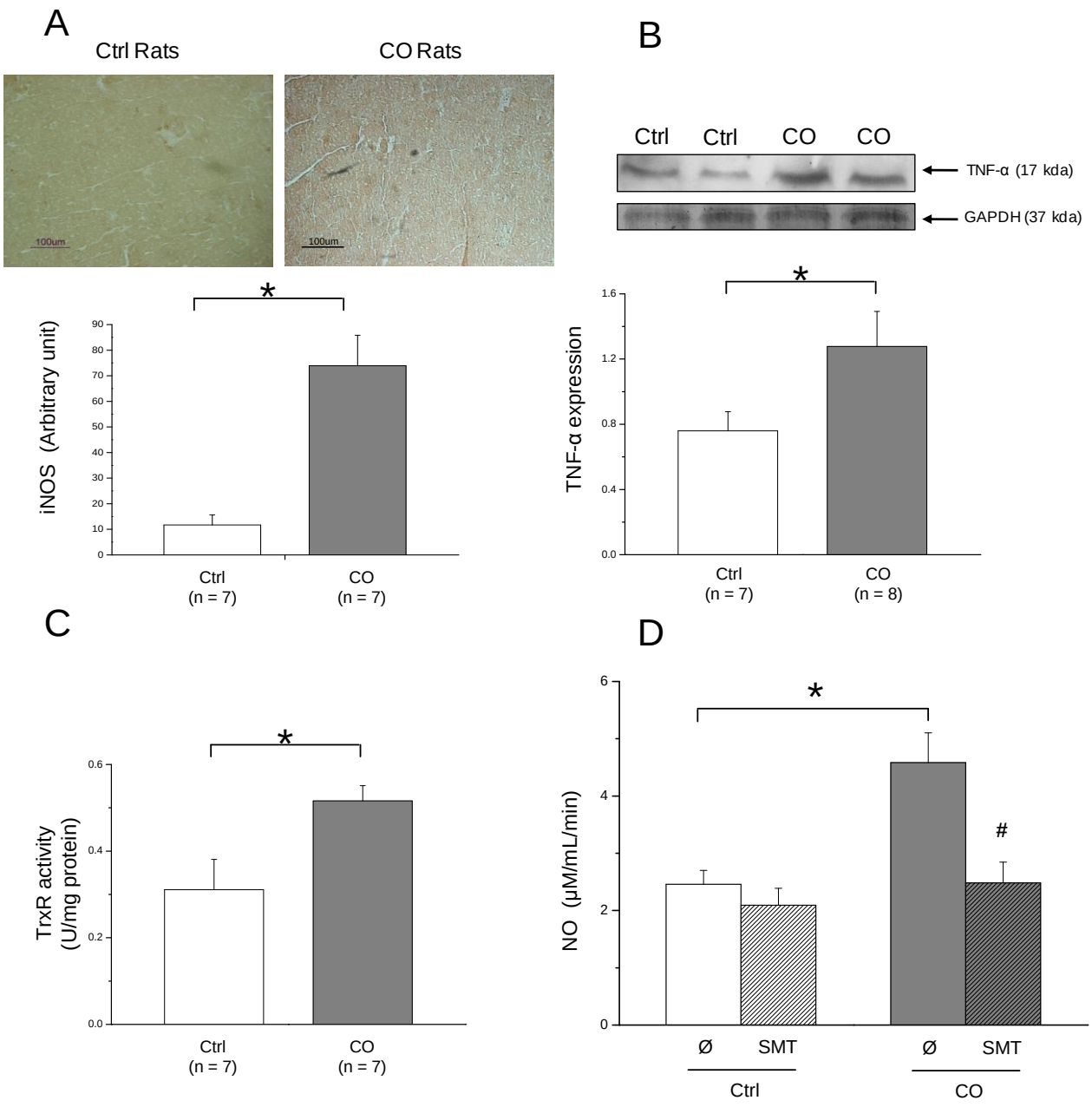

A
Ctrl Rats
CO Rats
100um
100um
iNOS (Arbitrary unit)
Ctrl
(n = 7)
CO
(n = 7)
B
Ctrl
Ctrl
CO
CO
TNF-α (17 kda)
GAPDH (37 kda)
TNF-α expression
Ctrl
(n = 7)
CO
(n = 8)
C
TrxR activity
(U/mg protein)
Ctrl
(n = 7)
CO
(n = 7)
D
NO (µM/mL/min)
Ø
SMT
Ø
SMT
Ctrl
CO
#

Fig. 2

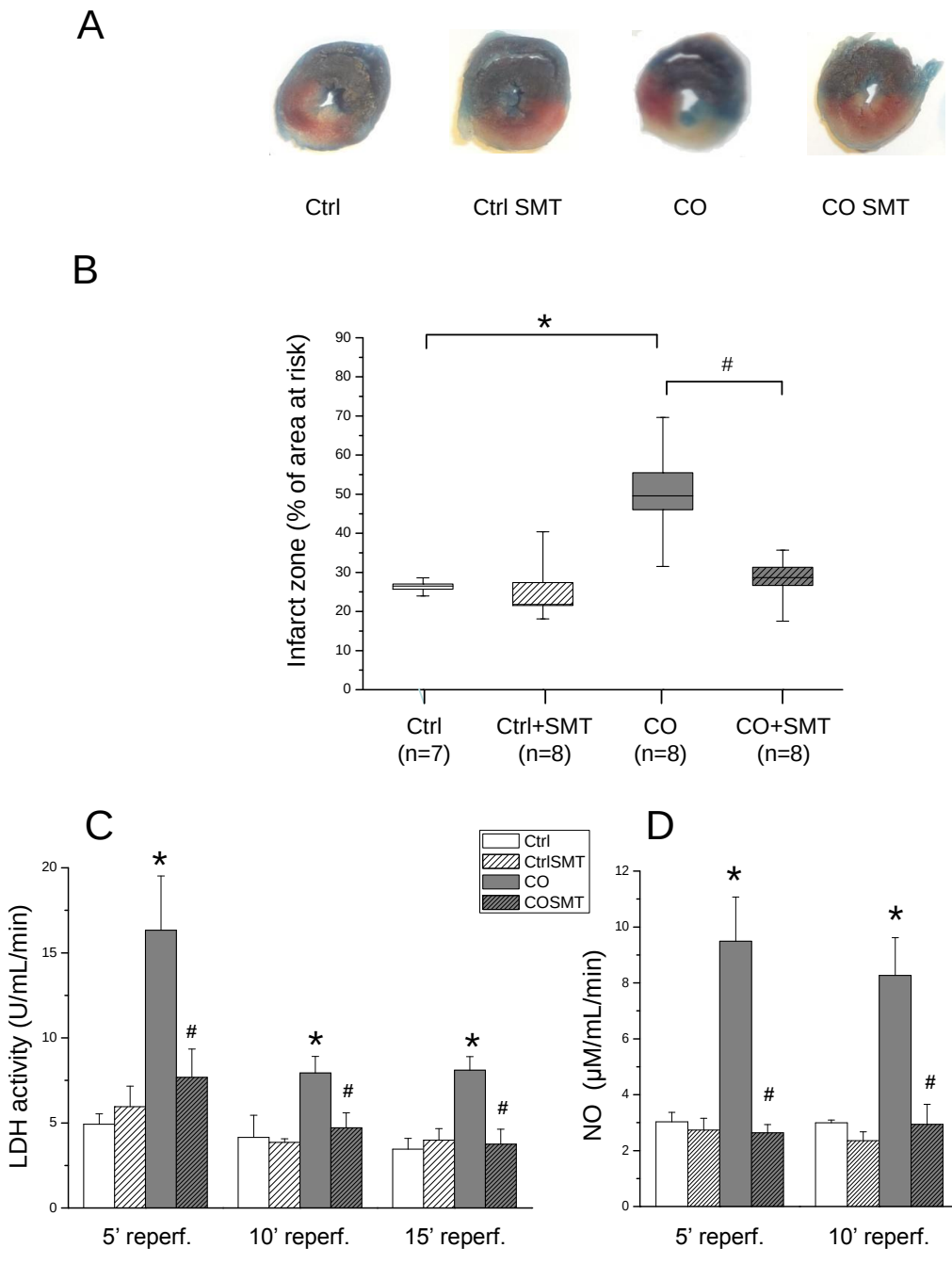
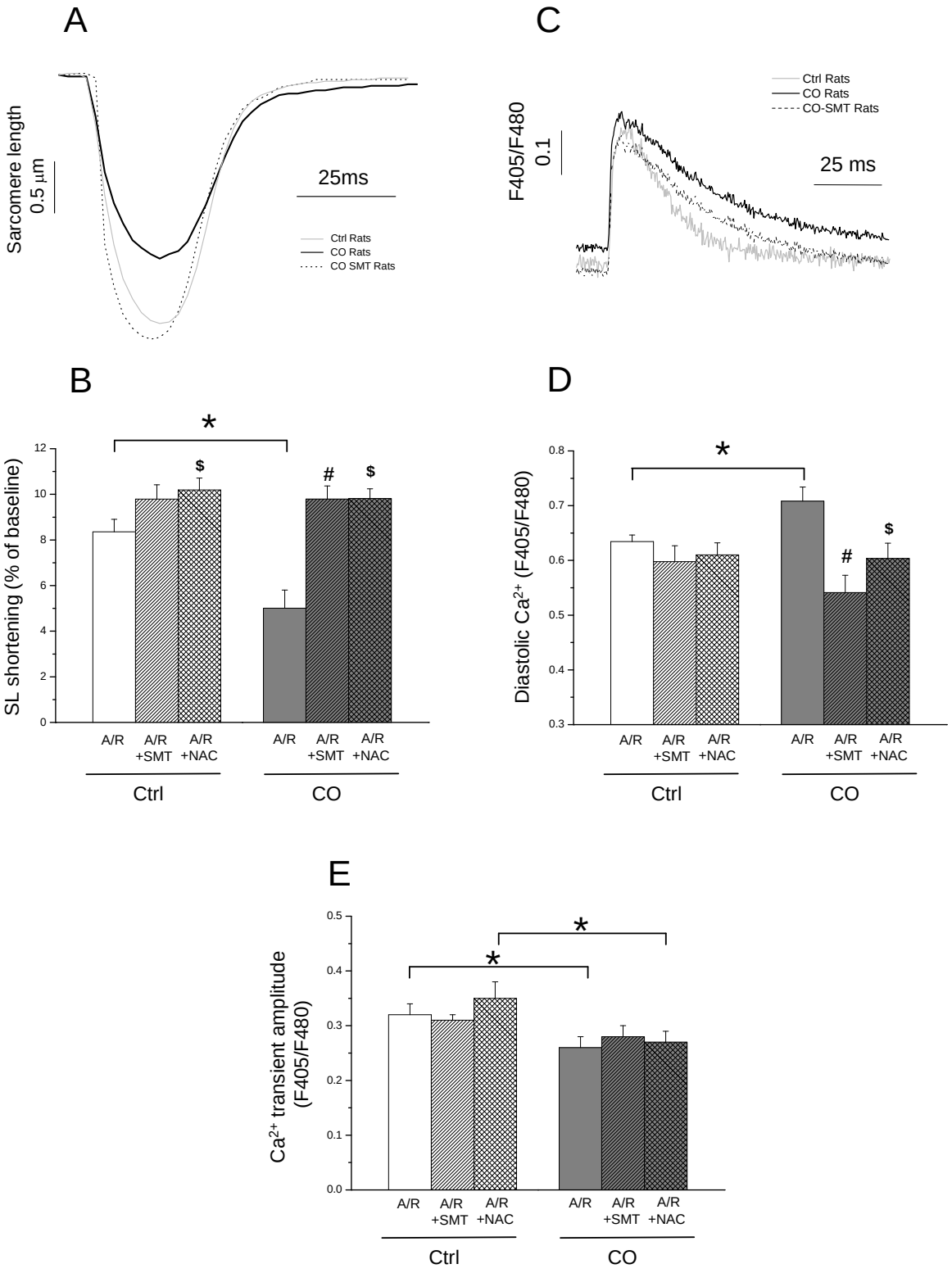

A
Ctrl
Ctrl SMT
CO
CO SMT
B
Infarct zone (% of area at risk)
Ctrl (n=7)
Ctrl+SMT (n=8)
CO (n=8)
CO+SMT (n=8)
C
LDH activity (U/mL/min)
Ctrl
CtrlSMT
CO
COSMT
5' reperf.
10' reperf.
15' reperf.
D
NO (µM/mL/min)
5' reperf.
10' reperf.
15' reperf.

Fig. 3


A
Sarcomere length
0.5 µm
25ms
Ctrl Rats
CO Rats
CO SMT Rats
C
F405/F480
0.1
25 ms
Ctrl Rats
CO Rats
CO-SMT Rats
B
SL shortening (% of baseline)
*
$
#
$
A/R
A/R +SMT
A/R +NAC
Ctrl
CO
D
Diastolic Ca2+ (F405/F480)
*
#
$
Ctrl
CO
E
Ca2+ transient amplitude (F405/F480)
*
*
Ctrl
CO