

Chronic Cocaine-Induced Cardiac Oxidative Stress and Mitogen-Activated Protein Kinase Activation: The Role of Nox2 Oxidase

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ABSTRACT

Chronic cocaine exposure is associated with severe cardiac complications, but the mechanisms of cocaine cardiotoxicity remain unclear, and current therapies are unsatisfactory. We investigated the hypothesis of oxidative stress-mediated cardiotoxicity and the role of NADPH oxidase in this process in a mouse model of chronic escalating "binge" cocaine administration (milligrams per kilogram): days 1 to 4 at 3×15 mg, days 5 to 8 at 3×20 mg, days 9 to 12 at 3×25 mg, and days 13 to 14 at 3×30 mg. Compared with vehicle controls, chronic binge cocaine administration significantly increased the cardiac NADPH-dependent O_2^- production (1.96 ± 0.4 -fold) as detected by tiron (an O_2^- scavenger)-inhibitable lucigenin chemiluminescence and dihydroethidium fluorescence. Cocaine-induced reactive oxygen species (ROS) production was associated with significant increases (~ 2 -fold) in the protein expressions of Nox2 (an isoform of NADPH oxidase) and its regulatory subunits: p22^{phox}, p67^{phox}, p47^{phox}, p40^{phox}, and Rac1, and in p47^{phox} phosphorylation as detected by immuno-

blotting (all $p < 0.03$). Increased Nox2 activity was accompanied by the activation of extracellular signal-regulated kinase 1/2, p38 mitogen-activated protein kinase (MAPK), and c-Jun NH₂-terminal kinase, notably in the cardiomyocytes. Cell culture experiments revealed that cocaine-induced ROS production was primarily a direct action of cocaine on cardiac myocytes, which caused severe oxidative damage to myocytes and cell death as detected by terminal deoxynucleotidyl transferase dUTP nick-end labeling assay. These could be inhibited by inhibitors to protein kinase C (bisindolymaleimide) or by depletion of Nox2 using small interfering RNA. In conclusion, chronic cocaine administration directly causes severe myocardial oxidative stress through the activation of Nox2 oxidase. Increased ROS production contributes to MAPK activation and the subsequent myocyte damage. Inhibitors to NADPH oxidase or antioxidants may have therapeutic potential in the treatment of cocaine cardiotoxicity.

Cocaine is one of the most common illicitly used drugs in the world and causes the most frequent drug-related deaths in young adults (<40 years) (Vasica and Tennant, 2002; Darke et al., 2006; Afonso et al., 2007). Chronic cocaine consumption is associated with serious cardiovascular complications such as hypertension, cardiac hypertrophy, and sudden death (Vasica and Tennant, 2002; Darke et al., 2006; Afonso et al., 2007). Conventionally, cocaine cardiotoxicity has been thought to be mediated indirectly through its sympathomimetic effect, i.e., by inhibiting the reuptake and in-

creasing the levels of neuronal catecholamines to work on adrenoceptors (Afonso et al., 2007). However, clinical therapies with adrenoceptor antagonists are problematic and not always effective (Vasica and Tennant, 2002; Afonso et al., 2007).

It has been discovered recently that cocaine administration is associated with severe oxidative stress in the heart (Moritz et al., 2003; Pacifici et al., 2003; Kovacic, 2005; Ren et al., 2006; Isabelle et al., 2007). Although there are several potential enzymatic sources of ROS existing in the heart, in an animal model of chronic cocaine administration, treatment with apocynin (an NADPH oxidase inhibitor) has been reported to be effective in reducing ROS generation and to restore the cardiac output, stroke volume, and fractional shortening, suggestive of the involvement of an NADPH oxidase (Isabelle et al., 2007). However, apocynin has been reported recently to be not a selective NADPH oxidase inhib-

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ABBREVIATIONS: ROS, reactive oxygen species; MAPK, mitogen-activated protein kinase; DHE, dihydroethidium; ERK, extracellular signal-regulated kinase; JNK, c-Jun NH₂-terminal kinase; Bis, bisindolymaleimide; siRNA, small interfering RNA; PBS, phosphate-buffered saline; PKC, protein kinase C; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling; L-NAME, *N*- ω -nitro-L-arginine methyl ester.

itor but an antioxidant (Heumüller et al., 2008). Therefore, the enzymatic sources of cocaine-induced cardiac oxidative stress remain to be elucidated.

NADPH oxidase comprises a cytochrome *b*, which can be further divided into one catalytic subunit (a member of the Nox family) and one p22^{phox} and at least four regulatory subunits (p47^{phox}, p67^{phox}, p40^{phox}, and rac1). To date, five members of the Nox family have been identified (Nox1–5), each encoded by a separate gene with a different function (Sumimoto et al., 2005). Cardiac tissue expresses both Nox2 and Nox4 (Byrne et al., 2003; Ribé et al., 2008).

In this study, we used an experimental mouse model of chronic escalating dose “binge” cocaine administration to mimic the common pattern of human repeated cocaine consumption, where steady increases in drug dose are required to achieve the desired drug effect because of an increase in cocaine tolerance. We have investigated: 1) the effect of chronic cocaine administration on cardiac ROS production and the role of Nox2 and Nox4 in this process and 2) the mechanisms and the downstream signaling pathways of cocaine-induced NADPH oxidase activation. We have also examined the direct effect of cocaine on Nox2 activation and subsequent mitogen-activated protein kinase (MAPK) activation in cultured cardiac myocytes.

Materials and Methods

Reagents. Dihydroethidium (DHE) was purchased from Invitrogen (Carlsbad, CA). Polyclonal antibodies against p22^{phox}, Nox4,

p40^{phox}, p47^{phox}, p67^{phox}, rac1, and cardiac-troponin I were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Rabbit polyclonal antibodies against a 30-amino acid C-terminal fragment of Nox2 (Pep37), were kindly provided by Dr. F. Wientjes (University College London, UK) (Li and Shah, 2002). Antibodies to phospho-ERK1/2, phospho-p38 MAPK, phospho-JNK, and phosphoserine were from Cell Signaling Technology Inc. (Danvers, MA). Bisindolymaleimide (Bis) was from Calbiochem (San Diego, CA). All other chemicals were from Sigma Chemical (Poole, Dorset, UK), unless stated otherwise.

Animal Model of Chronic Escalating Dose Binge Cocaine Consumption. A mouse model (C57BL/6J, male, 5 weeks old) of chronic escalating dose binge cocaine administration was generated as described previously (Bailey et al., 2007). All studies were performed in accordance with protocols approved by the Home Office under the Animals (Scientific Procedures) Act 1986 UK. The control group was given intraperitoneal injections of saline (10 ml/kg), and the cocaine group was given cocaine dissolved in saline (10 ml/kg) three times daily at 1-h intervals. The first injection was given ~1 h after the lights were switched on. The cocaine doses were escalated in a pattern of 3×15 mg/kg for days 1 to 4, 3×20 mg/kg for days 5 to 8, 3×25 mg/kg for days 9 to 12, and 3×30 mg/kg for days 13 to 14 to mimic a common pattern of human cocaine abuse. Animals were killed 30 min after the last injection. Left ventricular tissues were used for measuring O₂⁻ generation, immunoblotting, and immunocytochemistry. Twelve mice from each group were used for the study.

Cardiac Myocyte Culture, Nox2 siRNA Transfection, and Cocaine Stimulation. The rat cardiac myocyte cell line (H9C2) was obtained from the American Type Culture Collection (Manassas, VA) and grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. For the stimulation with cocaine, cells

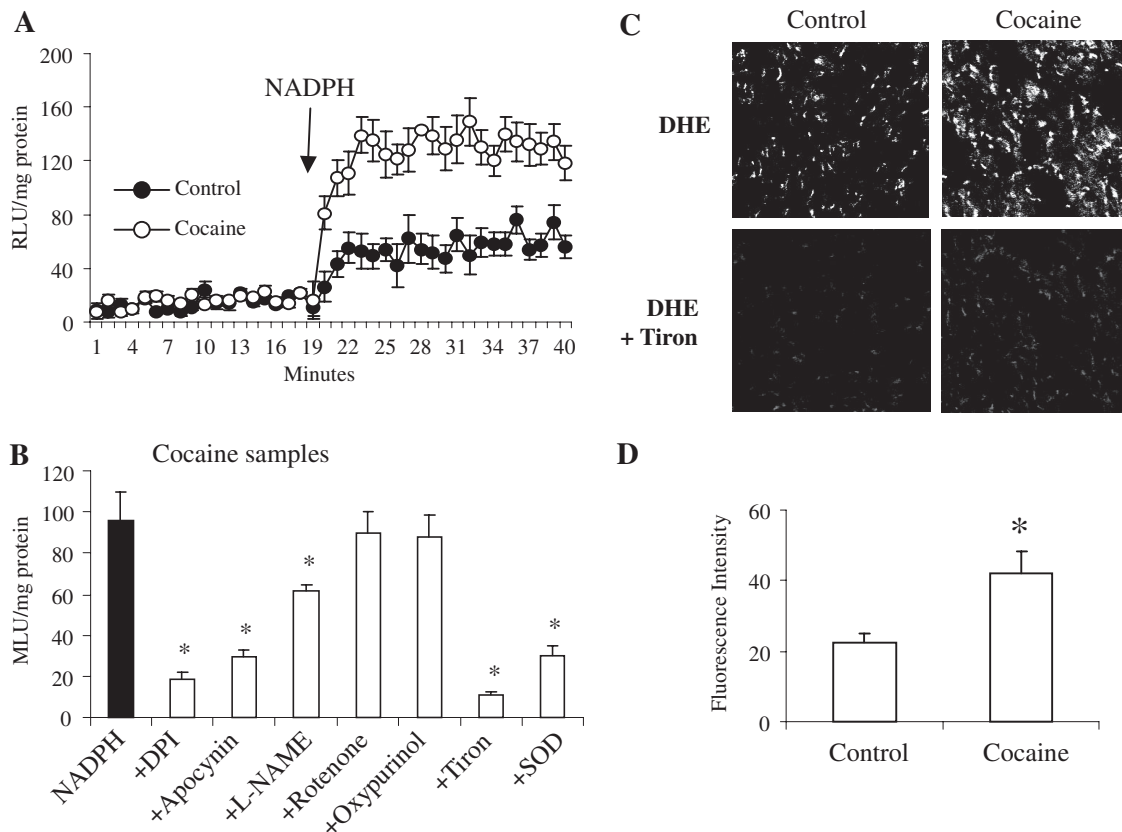


Fig. 1. O₂⁻ production in saline- and cocaine-treated hearts. A, kinetic detection of O₂⁻ production by lucigenin chemiluminescence. NADPH was added at 20 min of measurement. RLU, relative light unit(s). B, effects of inhibitors on the levels of O₂⁻ production by cocaine-treated hearts. *, $p < 0.05$ for indicated values versus the NADPH (without inhibitor) values. $n = 12$ hearts. C, tiron-inhibitable DHE fluorescence detection of ROS production by confocal microscopy. D, fluorescence intensities were quantified and expressed as mean \pm S.D. from 18 sections of six mice/per group. *, $p < 0.05$ for indicated values versus the controls.

were seeded the day before experiment at $8 \times 10^4/\text{ml}$ to achieve ~90% confluence and stimulated with cocaine (0–100 μM) in culture medium for 24 h in the absence or presence of a pan-PKC inhibitor, Bis (10 μM), or tirion (2.5 mM). Cells were washed three times with PBS and detached by scraping and snap-freezing in liquid nitrogen. In some experiments, cells were transfected with Nox2 siRNA or a random negative control siRNA with the nucleotide sequence exactly as described previously (Hingtgen et al., 2006) using Lipofectamine 2000 plus (Invitrogen, Paisley, UK) as transfection reagent (Li et al., 2007). Forty-eight hours after the transfection, cells were stimulated with cocaine (25 μM) for 24 h.

Measurement of ROS Production. O_2^- production by tissue or cell homogenate was measured using lucigenin (5 μM)-chemiluminescence (BMG Labtech GmbH, Offenburg, Germany) (Ribé et al., 2008). The specificity of the assay was confirmed by adding superoxide dismutase (100 units/ml) or tirion (10 mM). Other enzymatic sources of O_2^- production were identified using inhibitors such as L-NAME (100 μM), rotenone (50 μM), oxypurinol (100 μM), and diphenyleiiodonium (20 μM). All studies were performed in triplicate. ROS generation in cardiac section was measured in situ using DHE (1 μM) fluorescence in the presence or absence of tirion (Ribé et al., 2008). Fluorescence intensity was quantified under confocal microscopy from at least five random fields (1024×1022 pixels; $269.7 \times 269.2 \mu\text{m}$) per slide, three slides per animal and six animals per group.

Immunoprecipitation and Immunoblotting. Immunoprecipitation and immunoblotting was performed as described previously (Ribé et al., 2008). For p47^{phox} serine phosphorylation, the p47^{phox} was firstly immunoprecipitated down with antibodies to p47^{phox} coupled to protein G agarose beads overnight at 4°C. Normal rabbit IgG was used as a negative control. p47^{phox} serine phosphorylation

was detected by immunoblotting using a phosphoserine-specific monoclonal antibody.

Confocal Microscopy. Confocal microscopy were performed as described previously (Ribé et al., 2008). Biotin-conjugated anti-rabbit or anti-goat IgG were used as secondary antibodies and detected by extravidin-fluorescein isothiocyanate or streptavidin-Cy3. Normal rabbit or goat IgG (5 $\mu\text{g}/\text{ml}$) were used instead of primary antibody as a negative control. Images were acquired on a Zeiss LS510 confocal microscopy system (Carl Zeiss GmbH, Jena, Germany). Optical sections were taken at 1- μm intervals, and images were captured digitally.

TUNEL Assay. H9C2 cells were cultured onto the chamber slide and stimulated with cocaine (25 μM) for 24 h. The cells were then washed and fixed in 4% methanol-free formaldehyde/PBS solution and treated with 0.2% Triton X-100/PBS solution for 5 min. The TUNEL assay was performed using the DeadEnd fluorometric technique (Promega, Madison, WI) as described by the company and visualized under confocal microscopy.

Statistics. Data were presented as mean \pm S.D. Animal data were from 12 mice per group. Comparisons were made by unpaired Student's *t* test, with Bonferroni correction for multiple testing. *p* < 0.05 was considered statistically significant.

Results

Changes in the Levels of Cardiac ROS Production.

The O_2^- production in the cardiac homogenate was detected by lucigenin chemiluminescence (Fig. 1, A and B). There was no significant difference in the basal levels (without adding NADPH) of ROS production between saline-treated (control) and cocaine groups. However, the levels of NADPH-depen-

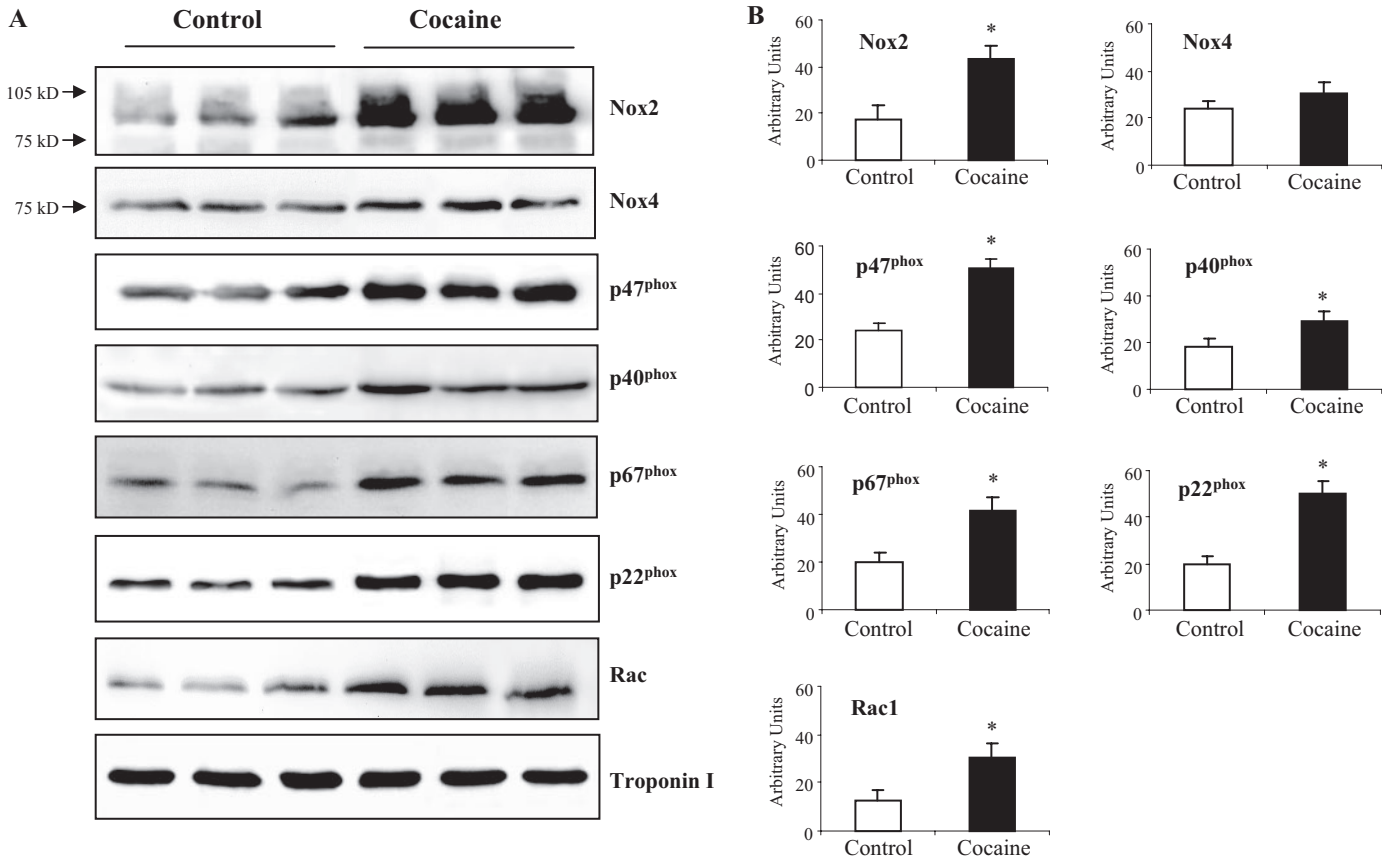


Fig. 2. Immunoblotting for the expression of NADPH oxidase subunits in saline- and cocaine-treated hearts. A, representative examples of immunoblots. Cardiac troponin I was used as a loading control. B, protein bands were quantified densitometrically and normalized to the expression of cardiac troponin I in the same sample. The results were expressed as arbitrary units. *, *p* < 0.05 for cocaine value versus saline controls. *n* = 12 animals.

dent O_2^- production were significantly increased in cocaine-treated hearts up to 1.96 ± 0.4 -fold of the controls ($p < 0.03$). The O_2^- production thus measured was virtually abolished by tiron (a superoxide scavenger), significantly inhibited by apocynin ($69 \pm 4\%$, NADPH oxidase inhibitor), diphenyleneiodonium ($82 \pm 2\%$, flavoprotein inhibitor), and superoxide dismutase ($69 \pm 4\%$) (all $p < 0.03$) but not by rotenone (mitochondrial complex 1 enzyme inhibitor) or oxypurinol (xanthine oxidase inhibitor) (Fig. 1B). There was a slight ($26 \pm 3\%$) but significant ($p < 0.05$) reduction in NADPH-dependent ROS production in the presence of L-NAME (NOS inhibitor) in cocaine-treated hearts. Although NOS uncoupling may account for a small proportion of increase in O_2^- generation in cocaine group, NADPH oxidase certainly represents a major source of chronic binge cocaine administration-induced O_2^- generation in the hearts.

The increase in O_2^- production was further examined by DHE fluorescence on cardiac sections in the presence or absence of tiron (Fig. 1C). Compared with saline controls, the tiron-inhibitable DHE fluorescence intensity in the myocardium of the cocaine-treated group was significantly increased to 1.83 ± 0.6 -fold compared with control values ($p < 0.05$) as quantified by confocal microscopy (Fig. 1D).

Changes in the Levels of NADPH Oxidase Expression. We then investigated the changes in the protein expression of NADPH oxidase subunits by immunoblotting (Fig. 2A). Results were normalized to the expression of cardiac troponin I detected in the same sample and quantified (Fig. 2B). Both Nox2 and Nox4 were detected in the hearts. In cocaine-treated hearts, there was a significant increase in Nox2 expression (2.34 ± 0.6 -fold compared with controls, $p < 0.05$) but not in Nox4 expression. Accompanying the increase in Nox2 expression, there were significant increases in p22^{phox} (2.5 ± 0.29 -fold), p67^{phox} (2.1 ± 4.1 -fold), p47^{phox} (2.6 ± 0.4 -fold), p40^{phox} (1.66 ± 0.32 -fold), and Rac1 (2.1 ± 0.37 -fold) compared with the control values (all $p < 0.05$). Because p47^{phox}, p67^{phox} and rac1 are only required for Nox2 but not Nox4 oxidase activation (Martyn et al., 2006), it is likely that Nox2 plays a prominent role in mediating cardiac oxidative stress in the cocaine group.

Changes in p47^{phox} Phosphorylation and Translocation. p47^{phox} phosphorylation has been shown to be a prerequisite for Nox2 oxidase activation (Li and Shah, 2003). Therefore, we examined the serine phosphorylation of p47^{phox} by immunoblotting using a phosphoserine-specific monoclonal antibody after immunoprecipitation of p47^{phox} (Fig. 3A). We found that chronic cocaine administration increased the levels of p47^{phox} serine phosphorylation by 3.2 ± 0.5 -fold compared with control levels ($p < 0.03$). The increase in p47^{phox} expression (Cy3-labeled) was notably in the cardiac myocyte sarcolemmal membrane area (fluorescein isothiocyanate-labeled laminin) (Fig. 3B), and this was further highlighted by the yellow fluorescence in the superposed image of the cocaine group (Fig. 3B).

Changes in MAPK Activation. MAPK are important redox-signaling molecules involved in the pathogenesis of many cardiac diseases such as hypertrophy and heart failure (Bueno and Molckentin, 2002; Li et al., 2002a). We investigated the changes in the levels of ERK1/2, p38 MAPK and JNK activation using phosphorylation-specific monoclonal antibodies. The expression of total ERK1/2, p38 MAPK, and JNK in the same samples was used as loading controls (Fig.

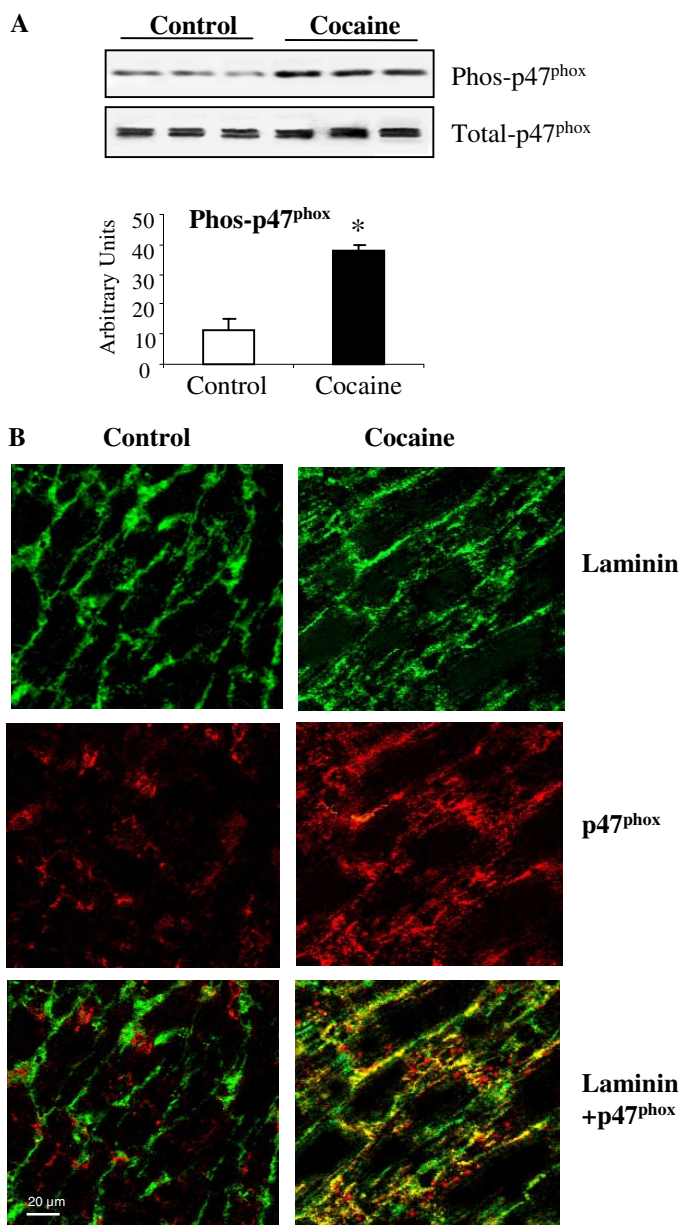


Fig. 3. p47^{phox} phosphorylation and myocyte expression of p47^{phox} in saline- and cocaine-treated hearts. A, immunoblotting. Phos-p47^{phox} bands were quantified densitometrically and normalized to the total p47^{phox} level in the same sample and expressed as arbitrary units. $n = 12$ hearts. *, $p < 0.05$ for cocaine values versus saline control values. B, confocal microscopy for the expression of p47^{phox} in cardiac myocytes. Cardiac myocytes were outlined by laminin (green fluorescence) and detected for the expression of the p47^{phox} (red fluorescence).

4A). We found that the levels of phosphorylated ERK1/2, p38 MAPK, and JNK in cocaine-treated hearts were significantly increased by 2.7 ± 0.4 -, 2.5 ± 0.25 -, and 2.6 ± 0.36 -fold, respectively, compared with saline controls (all $p < 0.05$).

Cocaine-induced ERK1/2 activation was further investigated by confocal microscopy on cardiac cryosections (Fig. 4B). Compared with saline-treated controls, there was a remarkable increase in phos-ERK1/2 expression in the sarcolemmal membrane area of the cardiomyocytes in the cocaine-treated group.

The Action of Cocaine on Cultured Cardiomyocytes. Conventionally, cocaine was believed to exert cardiotoxicity

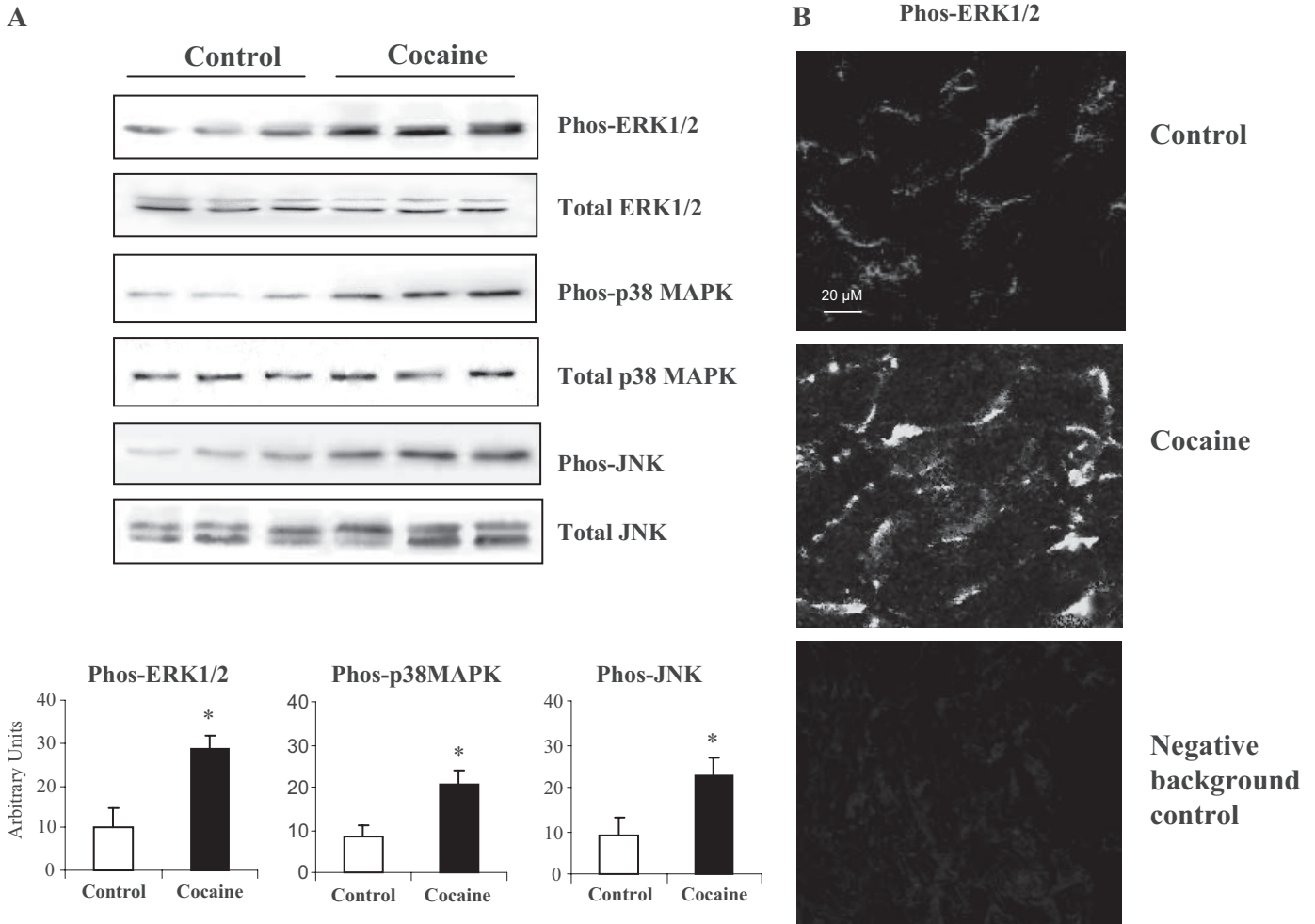


Fig. 4. Changes in MAPK activation in saline- and cocaine-treated hearts. A, immunoblotting. The phosphoprotein bands were quantified densitometrically and the levels of phos-ERK1/2, phos-p38 MAPK, and phos-JNK were normalized to the total protein levels of these molecules in the same samples and expressed as arbitrary units. $n = 12$ hearts. *, $p < 0.05$ for cocaine values versus saline values. B, confocal microscopy detection of phos-ERK1/2 in cardiac sections.

through an indirect sympathomimetic action on α -adrenergic receptors. However, cocaine also has been found to act directly on cardiomyocytes and damage cell function. To address the question of whether the action of cocaine on myocardial oxidative stress was direct or indirect, we stimulated H9C2 cells in culture with different concentrations of cocaine (0–100 μM) for 24 h and then examined the NADPH-dependent O_2^- production (Fig. 5A). Compared with control cells (without cocaine), cocaine used at a dose as low as 5 μM significantly increased O_2^- production up to 1.8 ± 0.3 -fold compared with control value ($p < 0.05$). At a dose of 25 μM or above, cocaine markedly increased the levels of O_2^- production up to a maximum of 3-fold compared with controls. Cocaine (25 μM)-induced ROS production by H9C2 cells was not significantly changed in the presence of a β -adrenergic receptor antagonist (propranolol, 1 μM) (Fig. 5B). However, cocaine-induced O_2^- production was accompanied by a remarkable increase in Nox2 protein expression and was virtually abolished by in vitro knockout of Nox2 expression using Nox2 siRNA (Fig. 5C). We then examined the effect of cocaine on myocyte death by TUNEL assay (Fig. 5D). Compared with control cells, cocaine (25 μM) caused severe myocyte damage and cell death as indicated by TUNEL-positive nuclei (yellow color), plus nuclear disintegration and fragmentation (arrow

in Fig. 5D). Cocaine-induced myocyte death was effectively inhibited by knockout of Nox2 using siRNA.

The Effects of the PKC Inhibitor on Cocaine-Induced Nox2 Activation. To investigate the possible mechanisms involved in the direct effect of cocaine on Nox2 activation, we examined the phosphorylation of p47^{phox} (a key regulator of Nox2 oxidase), p47^{phox} /Nox2 complex formation, and Nox2 oxidase activity (O_2^- production) in cultured cardiac myocytes in the presence or absence of a pan-PKC inhibitor (Bis, 10 μM) (Fig. 6A). Cocaine (25 μM) significantly increased p47^{phox} serine phosphorylation, which was accompanied by a significant increase in p47^{phox} /Nox2 complex formation and in Nox2 oxidase activity (O_2^- production). All of these cocaine-induced effects could be significantly inhibited in the presence of Bis.

We then examined the effects of PKC inhibitor (Bis) and O_2^- scavenger (tiron, 2.5 mM) on cocaine-induced ERK1/2 phosphorylation (Fig. 6B). Compared with cells cultured in the medium (control), cocaine (25 μM) significantly increased the ERK1/2 phosphorylation, and this was significantly inhibited to the control levels in the presence of Bis or completely abolished in the presence of tiron. Taken together, these results implicated a critical role of PKC in mediating the direct effect of cocaine on Nox2 activation and further con-

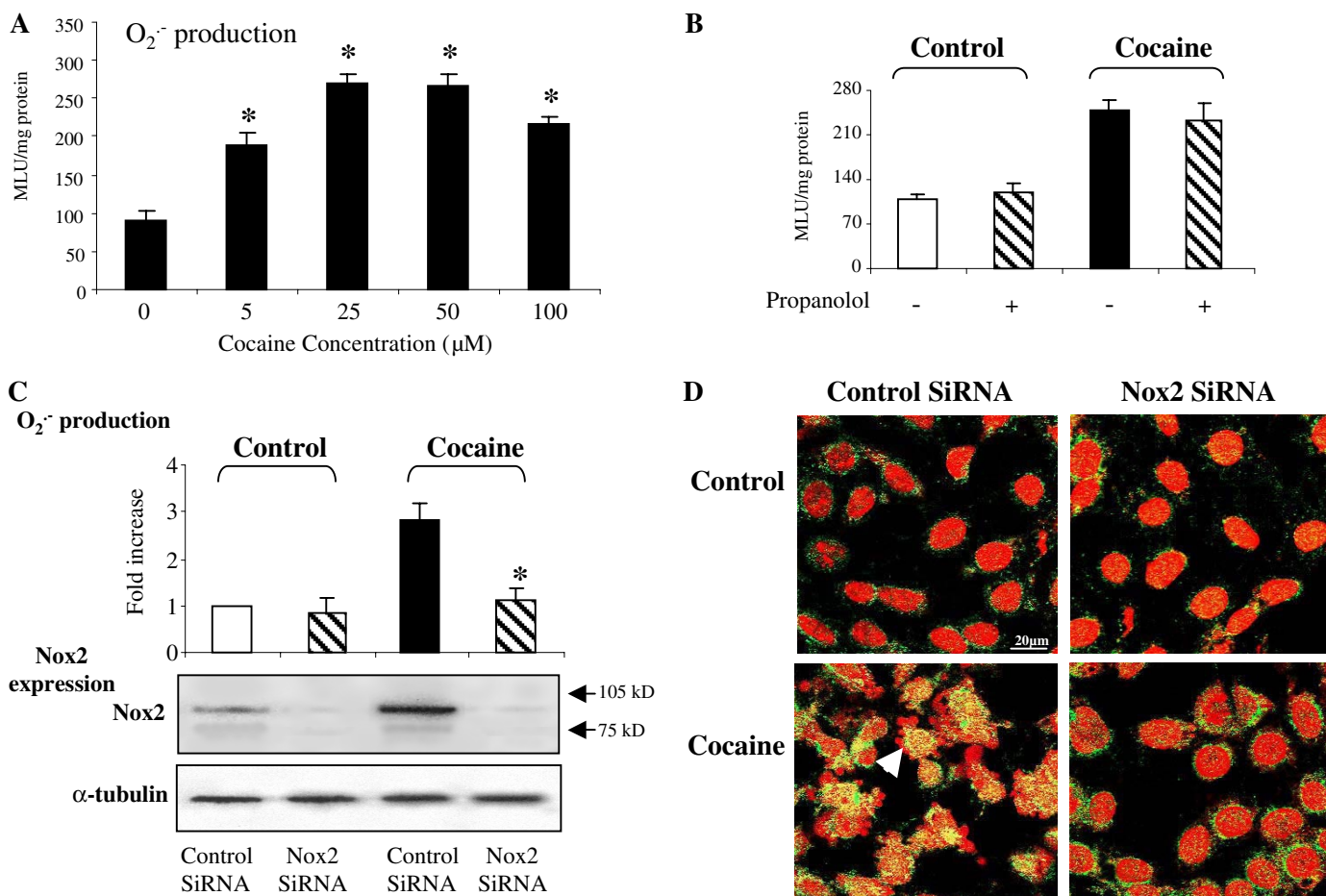


Fig. 5. Direct effects of cocaine on cultured H9C2 cardiomyocytes. **A**, cocaine-induced O_2^- production detected by lucigenin chemiluminescence. MLU, mean light unit(s). **B**, effect of propranolol on O_2^- production with or without cocaine ($25 \mu\text{M}$). **C**, effect of Nox2 siRNA on cocaine-induced O_2^- production and Nox2 protein expression. α -Tubulin was used as loading control. *, $p < 0.05$ for indicated values versus control values in the same group. **D**, effect of Nox2 siRNA on cocaine-induced myocyte death. Arrow, nuclear fragmentation and TUNEL-positive nucleus.

firmed the role of Nox2-derived ROS in cocaine-induced MAPK activation.

Discussion

Chronic cocaine consumption has been shown to be associated with life-threatening cardiac abnormalities including left ventricular hypertrophy, arrhythmias, heart failure, myocarditis, and sudden cardiac death (Vasica and Tennant, 2002; Darke et al., 2006; Afonso et al., 2007). However, our understanding of the underlying mechanisms of cocaine cardiotoxicity is far from complete and pharmacological management of such patients is problematic (Vasica and Tennant, 2002; Afonso et al., 2007). In this study, we used an experimental mouse model of chronic escalating dose of cocaine binge administration to mimic the common pattern of human repeated cocaine consumption. We found that chronic cocaine consumption significantly increased the cardiac NADPH-dependent O_2^- generation because of an increase in the expression of Nox2 components and the p47^{phox} phosphorylation.

Emerging evidence has revealed that cardiac oxidative stress is a prominent early event of cocaine administration, which severely compromises the cardiac antioxidative system and causes cardiac damage (Boess et al., 2000; Kovacic, 2005). Evidences of oxidative damage such as peroxidation of membrane phospholipids and depletion of nonenzymatic an-

tioxidants such as GSH have been found in the myocardium of chronic cocaine-treated animals (Pacifci et al., 2003) and in patients (Darke et al., 2006; Afonso et al., 2007). In the present study, using two independent assays, we have provided direct evidence that chronic cocaine binge consumption doubles the cardiac O_2^- production. A small but significant proportion ($\sim 25\%$) of the NADPH-dependent ROS production was inhibited by an NOS inhibitor (L-NAME), indicating that dysfunctional NOS activity might also partly contribute to ROS production in cocaine group (Li and Shah, 2004). This is not surprising because ROS production from any source may induce dysfunctional O_2^- -generating NOS activity, as a consequence of ROS-dependent degradation of the essential NOS cofactor tetrahydrobiopterin (Vásquez-Vivar et al., 2003).

During the last decade, it has become clear that the myocardium expresses constitutively both Nox2 and Nox4 (Byrne et al., 2003). In general, Nox2 activity is dependent on the phosphorylation of p47^{phox} (a major regulatory subunit) and requires the presence of other cytosolic components, such as rac1 and p67^{phox} (Li et al., 2002b; Li and Shah, 2004; Sumimoto et al., 2005), whereas Nox4 seems not to exhibit a binding site to p47^{phox} (Sumimoto et al., 2005) and is not regulated by p47^{phox} or any other known regulatory components (Martyn et al., 2006). In the present study, we demonstrated by immunoblotting that chronic binge cocaine consumption sig-

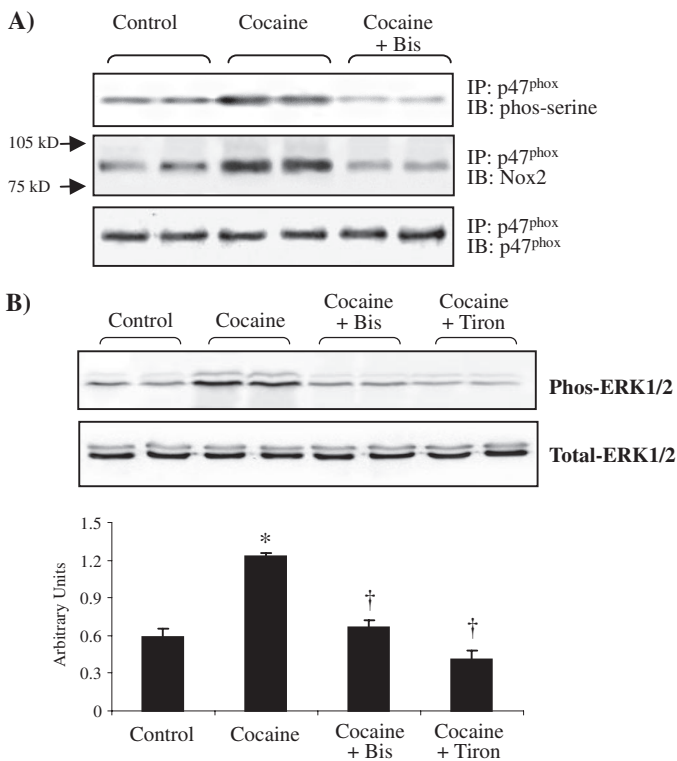


Fig. 6. The effects of PKC inhibitor and O_2^- scavenger on cocaine (25 μ M)-induced p47^{phox} serine phosphorylation and ERK1/2 activation in H9C2 cells. A, p47^{phox} was immunoprecipitated down (IP) and detected by immunoblotting (IB) using antibodies specific to phosphoserine or to Nox2. p47^{phox} IB was used as loading control. B, cocaine-induced ERK1/2 phosphorylation in H9C2 cells in the presence and absence of Bis or tiron. The phos-ERK1/2 bands were quantified densitometrically and normalized to the total ERK1/2 levels in the same samples and expressed as arbitrary units. *, $p < 0.05$ for cocaine values versus control (without cocaine) values. †, $p < 0.05$ for the values of cocaine plus Bis or tiron versus the cocaine values. $n = 4$ independent cell cultures.

nificantly up-regulated the protein transcription of Nox2 and its components: p22^{phox}, p47^{phox}, p67^{phox}, and rac1. The role of Nox2 in mediating cocaine oxidative stress was further supported by our data that cocaine treatment indeed increased p47^{phox} serine phosphorylation (up to 3-fold) in the cardiomyocytes as demonstrated by confocal microscopy.

ROS have multiple effects on cell function depending on the amount and subcellular location of ROS generated. Controlled intracellular ROS production from NADPH oxidase has been shown to be necessary for normal cellular development and function (Biswas et al., 2006), whereas excessive ROS generation is implicated in myocardial hypertrophy and heart failure (Li et al., 2002a). The mitogenic effects of ROS may involve the modulation of redox-sensitive signaling pathways such as the MAPK superfamily, which lead to the activation of transcriptional factors (Li et al., 2002a, 2004). MAPKs, in particular ERK1/2, have been shown to be activated by cocaine administration and represent an important cellular mechanism in cocaine addition (Lu et al., 2006). In accordance with previous reports, we found significant increases in the levels of ERK1/2, p38 MAPK, and JNK phosphorylation in cocaine-treated hearts. In addition, we also found, by confocal microscopy, that the increases in p47^{phox} expression and ERK1/2 phosphorylation were both in the sarcolemmal membrane area of cardiac myocytes in cocaine-treated hearts. Therefore, MAPKs can serve as intermediates

that couple Nox2 oxidase to its downstream signaling molecules involved in cocaine cardiotoxicity.

Cocaine-induced cardiac oxidative stress has been regarded previously as an indirect effect of cocaine on cardiomyocytes via its sympathomimetic action on adrenoceptors (Afonso et al., 2007; Isabelle et al., 2007). Although NADPH oxidase can also be activated by catecholamines, the severity of myocardial damage seen in cocaine patients could not be fully addressed by a simple sympathomimetic action. Moreover, the cardiac β -adrenoceptor signaling pathways are impaired by cocaine exposure (Sun, 2000), which is opposite to the conventional view of cocaine sympathomimetic action. The current study provides the first evidence that in the absence of catecholamines, cocaine used at a dose of 5 μ M, which is far below the plasma levels of cocaine found in cocaine abusers, has direct effects on cultured cardiac myocytes and strongly promotes ROS production from Nox2 oxidase. Cocaine, used at 25 μ M, a concentration that is relevant to the mean plasma concentration found in patients (Wu et al., 2006), caused a 3-fold increase in O_2^- generation and severe cell death in cultured cardiac myocyte. All these effects were abolished or effectively inhibited by in vitro knock-out of Nox2 using siRNA. Furthermore, using inhibitor of PKC and an O_2^- scavenger (tiron), we showed that the effect of cocaine on cardiac myocyte ROS production is PKC-dependent and an increase in O_2^- generation from Nox2 oxidase is a prerequisite for cocaine-induced ERK1/2 activation. It is well known that PKC-mediated p47^{phox} phosphorylation is a key step in Nox2 oxidase activation. Therefore, cocaine may act through PKC to cause p47^{phox} phosphorylation and to activate Nox2 oxidase rather than indirectly through the actions on adrenoceptors. However, we have not examined yet whether other PKC downstream signaling pathways are involved in cocaine cardiac toxicity. It is clear from the current study that cocaine can cause myocyte oxidative damage without adrenergic signals. However, in the in vivo situation, adrenergic signals can further escalate the severity of cocaine-related oxidative stress and toxicity to the heart. Nevertheless, it is important to know that knockout of Nox2 can effectively reduce cocaine-induced myocardial oxidative stress and damage.

In conclusion, we report for the first time that chronic cocaine administration causes severe cardiac oxidative stress through the activation of Nox2 oxidase. The mechanisms involved are the transcriptional up-regulation of Nox2 oxidase components and the post-translational modification of p47^{phox} phosphorylation. We also found that cocaine is able to cause p47^{phox} phosphorylation in cardiac myocytes through PKC, in the absence of catecholamines. Further investigation into the inhibition of p47^{phox} phosphorylation and Nox2 activation may provide a potential therapeutic strategy for cocaine cardiotoxicity.

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