Role of reactive oxygen species in cocaine-induced cardiac dysfunction

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Abstract

Objective: Contractility alterations and LV hypertrophy after chronic cocaine administration have been shown to be accompanied by an increase in oxidative stress. This study was carried out to investigate whether the production of reactive oxygen species is an early event of primary importance in cocaine-induced myocardial injury or simply occurs as a consequence of the ventricular dysfunction itself.

Methods and results: After 2 days of cocaine administration to rats, no differences were observed in echocardiographic parameters between the cocaine-treated group and the control group. However, an increase in oxidative stress in the myocardium was indicated by an increase in lipid peroxidation (+35%, cocaine vs. control), an increase in antioxidant enzymes (catalase +110%, glutathione peroxidase +40% and superoxide dismutase +38%) and of NADPH-driven superoxide production (assessed by chemiluminescence). Furthermore, higher gp91phox and p22phox mRNA expression, measured by quantitative real-time RT-PCR, was found in the cocaine group. On day 8, cocaine administration induced a cardiac dysfunction, characterized by a decrease in cardiac index (~30%, cocaine vs. controls) and left ventricular (LV) fractional shortening (~23%, cocaine vs. controls). This LV dysfunction was prevented by antioxidant treatment (100 mg/kg/day vitamin C and 100 U/kg/day vitamin E). Moreover, in these animals, antioxidant treatment decreased lipid peroxides and decreased the activity of NADPH oxidase, associated with the downregulation of gp91phox. Conclusion: These data indicate that cocaine administration induces early NADPH-driven O2 release which may play an important role in the development and progression of the LV dysfunction observed after chronic cocaine abuse.

Keywords: Cocaine; Free radicals; Ventricular function; Superoxide; Hypertrophy; Vitamin E; Vitamin C

1. Introduction

Cocaine-induced cardiovascular disorders such as hypertension, thrombosis, myocardial dysfunction, cardiac dysrhythmias and endocarditis have received widespread attention in the context of cocaine abuse [1–6]. The number of sudden deaths from cardiac causes, including myocardial infarction, ventricular tachyarrhythmia or aortic dissection, is also increasing [6]. Many reports have postulated a relationship between cocaine-induced cardiovascular events and catecholamine accumulation, due to both the stimulation of the sympathetic system and the blockage of norepinephrine reuptake [7,8]. On the other hand, catecholamine-induced cardiomyopathy is partially mediated by the production of radical oxygen species (ROSs) [9,10] and recent experimental studies have reported signs of oxidative stress in the myocardium of chronic cocaine-treated rats [11,12]. However, the question arises as to whether oxidative stress is an early triggering event of cocaine-induced myocardial injury, or simply occurs as a cellular response secondary to the ventricular dysfunction. Moreover, the exact source of the oxygen free radicals in cocaine-induce cardiotoxicity has never been elucidated.

Within the heart, possible enzymatic sources of ROS include mitochondrial respiration and the xanthine oxido-
reductase system, which have been implicated in ROS production during postischemic myocardial dysfunction (for a review, see Ref. [13]). More recently, it has been reported that neutrophil-type NAD(P)H oxidases are a major source of ROS in cardiovascular cells [14]. This enzymatic system is implicated in O$_2^-$ production in cardiac myocytes upon hypoxia or lactate supplementation [15] or after myocardial infarction [16], and plays an important role in angiotensin-II-induced cardiac hypertrophy [17].

To clarify the links between oxidative stress and cocaine-induced cardiac dysfunction, the purpose of our study was:

- to characterize whether cocaine induces the production of reactive oxygen species, occurring before any detectable contractile dysfunction, as well as the role of NADPH oxidase in this production; and
- to assess whether prevention of oxidative stress (by antioxidant treatment) affects cocaine-induced cardiac dysfunction in rats.

2. Methods

2.1. Experimental protocols

This study consisted of two protocols. These experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.1.1. Protocol 1

Twenty-four male Wistar rats (325–350 g; Iffa-Credo, L’Arbresle, France) were randomly allocated to receive saline (control group) or cocaine hydrochloride (cocaine group). Treatment regimens consisted of cocaine injections (2×7.5 mg/kg/day, i.p.; Sigma) administered for 1 day. On day 2, animals were anesthetized 3 h after a single cocaine (7.5 mg/kg) or saline injection for echocardiographic measurements and the heart was then removed from the chest for biochemical analysis.

2.1.2. Protocol 2

Forty-eight male Wistar rats (325–350 g; Iffa-Credo) were randomly divided into four groups, each consisting of 12 animals: control, cocaine, vitamin C plus vitamin E (VitCVitE group) and cocaine plus vitamin C plus vitamin E (COC+VitCvitE group). The animals in the control group were injected with saline. In the VitCvitE and COC+VitCvitE groups, vitamin E was administered orally through a gastric tube, in 0.5 ml corn oil, at a dose of 100 U/kg/day. Vitamin C was given in the drinking water, at a dose of 100 mg/kg/day. The dosages of vitamins C and E were based on previous studies [18,19]. Treatment with antioxidants was started 2 weeks before initiation of cocaine, and was continued during the 1 week of cocaine administration. Cocaine hydrochloride (2×7.5 mg/kg/day, i.p.) was administered for 7 days. On day 8, animals were anesthetized 3 h after a single cocaine (7.5 mg/kg) or saline injection for evaluation of cardiac function by echocardiography. The heart was then removed from the chest for biochemical analysis.

2.2. Hemodynamic measurements

Systemic blood pressure and heart rate were determined in conscious rats by plethysmography on days 2 and 8.

2.3. Echocardiographic measurements

The echocardiography measurements were performed blinded to the animal group. Two-dimensional and M-mode echocardiography was performed using a 5-MHz transducer and an ATL HDI 5000 echograph, in anaesthetized animals (midazolam 0.8 mg/kg and ketamine 350 mg/kg, i.p.). Briefly, LV M-mode tracings were recorded from a two-dimensional short-axis view of the left ventricle obtained at the level of the papillary muscle. Maximal LV end-diastolic dimensions (EDDs) and end-systolic dimensions (ESDs) were measured at the level of the papillary muscle and used to calculate LV fractional shortening (FS) using the equation $FS = [(EDD - ESD)/EDD] \times 100$ [20,21].

In addition, the LV outflow velocity was measured by pulsed-wave Doppler, and the cardiac index was calculated as $CI = \text{aortic VTI} \times [\text{LV outflow diameter} / 2]^2 \times \text{heart rate/body weight}$, where VTI is the velocity–time integral.

2.4. Antioxidant enzymes

The LV myocardium was dissected and homogenized in 5 mM Tris HCl, 0.9% NaCl, pH 7.4 for enzymatic measurements.

2.4.1. Glutathione peroxidase (GPX) assay

Glutathione peroxidase (GPX) activity was measured by continuous monitoring of the regeneration of GSH from GSSG by the action of glutathione reductase with NADPH [22]. Homogenates (20–25 mg proteins) were incubated at 37°C in a final volume of 250 μl with potassium phosphate (62.5 mM pH 7) containing 0.62 mM EDTA and 0.62 mM sodium azide and the following solutions: glutathione reductase (7.5 U/ml), reduced glutathione (12.5 mM), NADPH (3 mM). The reaction was initiated by the addition of 6 mM H$_2$O$_2$ and the conversion of NADPH to NADP was assayed by measuring the absorbance at 340 nm for 2 min. GPX activity was expressed as micromoles of NADPH oxidized to NADP per minute per milligram protein with a molar extinction coefficient for NADPH of 6.22×10$^3$ M$^{-1}$cm$^{-1}$. 

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2.4.2. Catalase (CAT) assay

Catalase (CAT) activity was measured spectrophotometrically by monitoring the decomposition of hydrogen peroxide [23]. The homogenate (50–100 μg proteins) was added to a cuvette containing potassium phosphate (50 mM, pH 7) with 25 mM H₂O₂. The total reaction mixture was 1 ml. The disappearance of H₂O₂ was monitored at 240 nm for 1 min at 25 °C. CAT activity was expressed as μmoles of H₂O₂ consumed per minute per milligram protein with a molar extinction coefficient of 43.6 M⁻¹·cm⁻¹.

2.4.3. Superoxide dismutase (SOD) assay

Total superoxide dismutase (SOD) activities were assayed by measuring the inhibition of xanthine plus xanthine oxidase mediated cytochrome c reduction [24]. In order to eliminate interference with cytochrome oxidase or peroxidase, 10 mM potassium cyanide was added to the reaction mixture containing 10 mM acetylated cytochrome c, 50 mM hypoxanthine and 8 mM xanthine oxidase in 50 mM potassium phosphate, 0.1 mM EDTA, pH 7.8. Changes in absorbance were followed for 2 min at 418 nm. SOD activity was expressed as units per milligrams protein.

2.5. Malondialdehyde (MDA) assay

The lipid peroxide content of the hearts was studied by determining the thiobarbituric acid reactive substances (TBARS) for the estimation of MDA content, as described by Dhalla [25]. The LV myocardium was dissected, homogenized (10% w/v) in 0.2 M Tris–HCl, 0.16 M KCl, pH 7.4 supplemented with 0.02% butylated hydroxytoluene and incubated for 1 h at 37 °C. After mixing, a 1.0 ml aliquot was withdrawn from the incubation mixture and placed in a Pyrex tube. This was followed by the addition of 1 ml of 40% trichloroacetic acid (TCA) and 1 ml of 0.2% thiobarbiturate sodium. Tubes were boiled for 30 min and cooled on ice. Two millimeters of 70% TCA were added and the tubes were then centrifuged at 800 g for 20 min. The supernatant was assayed by measuring the absorbance at 532 nm and the concentration of MDA was calculated from MDA standards. Results were expressed in ng of MDA mg⁻¹ heart tissue.

2.6. Measurement of O₂⁻ production in microsomal fractions

The microsomal fraction was prepared according to Mohazzab and Wolin [26], and adapted to the LV myocardium. Briefly, myocardial tissues were finely minced and homogenized on ice with a tissue homogenizer (ultraturax) in 20 mM monobasic potassium phosphate pH 7.0, 0.01 mM EDTA, and 250 mM saccharose buffer containing protease inhibitors (1 μg/ml aprotinin, 0.5 μg/ml leupeptine, 87 μg/ml phenylmethylsulfonyl fluoride). The fractions obtained from the 100 000 g pellets were used to evaluate the NADPH oxidase activity measured by superoxide-dependent lucigenin chemiluminescence. Microsomal fractions (0.05 and 0.1 mg/ml of protein) were added to a glass scintillation vial in 20 mM monobasic potassium phosphate pH 7.0, and 0.01 mM EDTA containing 5 μM lucigenin, an optimal concentration for assessing O₂⁻ in tissues [27]. The reaction was started by the addition of 500 μM NADPH to the incubation medium as a substrate for O₂⁻ production. Luminescence was measured in a dark room with a scintillation counter (Wallac 1410). Measurements were integrated for a 1 min period and the cycle repeated three times, then averaged. The background was determined by measurement in the absence of homogenate and then subtracted from the readings obtained in the presence of biological material. The following agents were used to assess the specificity of this assay: 50 μM NBT (nitroblue tetrazolium), 100 μM oxypurinol, a xanthine oxidase inhibitor, or 10 μM diphenyleneiodonium, a flavoprotein inhibitor.

2.7. RNA isolation and real-time RT-PCR

Total RNA was isolated from the LV myocardium according to the acidic/phenol/chloroform procedure [28]. DNase treatment was carried out to remove contaminating genomic DNA, as previously described [29]. Reverse transcriptase (RT) was performed for 1 h at 37 °C using 1 μg total RNA in the presence of 500 ng oligo(dT)₁₂–₁₈ (Amersham Pharmacia Biotech) and 400 U Moloney Murine Leukemia Virus Reverse Transcriptase (Life Technologies). Real-time PCR was performed on a LightCycler (Roche Molecular Biochemicals, Mannheim, Germany) using a commercially available mix containing Taq DNA polymerase, SYBR-Green I, and deoxyribonucleoside triphosphates (FastStart DNA Master SYBR Green I kit; Roche). The following primers were used: p22phox: 5'-GCT CAT CTG TCT GGA GTA-3' (sense) and 5'-ACG ACC TCA TCT GTC ACT GGA-3' (antisense); gp91phox: 5'-CAG GGG TTC CAG TGC GTG TTG CTC-3' (sense) and 5'-GTT ACA GGA ACA TGG GAC CCA CTA TCC ATT TCC AAG-3' (antisense). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as housekeeping gene: 5'-TCC ATG ACA ACT TTG GCA-3' (sense) and 5'-CAT GTC AGA TCC ACC ACG GA-3' (antisense). Samples were analyzed in three independent runs. Serial dilutions of cDNA from total RNA were performed for each target gene. These served as standard curves for quantitative analysis. After the addition of primers (final concentrations 0.5 μM), MgCl₂ (4 mM) and template DNA to the master mix, 40 cycles of denaturation (95 °C for 15 s), annealing (64 °C, 10 s for p22phox and 62 °C, 10 s for GAPDH), and extension (72 °C, 17 and 15 s for p22phox and GAPDH, respectively) were performed. Detection of the fluorescent products was carried out at the end of the 72 °C extension.
period. To confirm amplification specificity, the PCR products were subjected to a melting curve analysis and subsequent agarose gel electrophoresis. Data were analyzed with the Light Cycler analysis software as described previously [30].

2.8. Statistical analysis

All results are expressed as mean±S.E.M. Data were analyzed using the Student test for unpaired data (protocol 1) and by analysis of variance (ANOVA) for multiple comparisons followed by the post hoc Tukey test (protocol 2) using Systat Software. Differences were considered to be statistically significant at P<0.05.

3. Results

3.1. Protocol 1: effects of short-term cocaine administration on oxidative stress

3.1.1. Echocardiography parameters

At the end of the cocaine treatment (day 2), the left ventricular (LV) fractional shortening, blood pressure and heart rate, the left ventricular end diastolic and systolic diameters (LVEDD and LVESD) as well as the cardiac index were not significantly modified (Table 1).

3.1.2. Antioxidant enzyme activities in cardiac homogenates

Myocardial catalase (CAT), glutathione peroxidase (GPX), and superoxide dismutase (SOD) activities evaluated after short-term cocaine administration are shown in Fig. 1. There was a significant increase in all these activities in cocaine-treated rats compared with their controls. Catalase activity was significantly increased by about 110% (Fig. 1A). GPX activity was increased by 40% (Fig. 1B) and a similar trend was seen with respect to MnSOD activity (+38%) (Fig. 1C).

Table 1

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Cocaine</th>
</tr>
</thead>
<tbody>
<tr>
<td>LVEDD (cm)</td>
<td>0.65±0.02</td>
<td>0.64±0.02</td>
</tr>
<tr>
<td>LVESD (cm)</td>
<td>0.37±0.02</td>
<td>0.37±0.02</td>
</tr>
<tr>
<td>Fractional shortening (%)</td>
<td>43±2</td>
<td>43±1</td>
</tr>
<tr>
<td>Cardiac index (ml.min⁻¹.kg⁻¹)</td>
<td>213±12</td>
<td>205±15</td>
</tr>
<tr>
<td>HR (beats/min)</td>
<td>397±9</td>
<td>376±9</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>137±3</td>
<td>131±3</td>
</tr>
</tbody>
</table>

LVEDD, left ventricular end-diastolic diameter; LVESD, left ventricular end-systolic diameter; HR, heart rate; SBP, systolic blood pressure (n=12 per group).

3.1.3. Malondialdehyde (MDA) content

We examined the MDA content of hearts as an estimation of lipid peroxidation (Fig. 2A). MDA levels significantly increased by 35% in cocaine-treated hearts compared with those of the control group.

3.1.4. NADPH-dependent superoxide production

The activity of NADPH oxidase, a major source of O₂⁻ in the cardiovascular system, was significantly greater in the cocaine group than in controls (about +80%) (Fig. 2B). To investigate the specificity of this increase, experiments were repeated with inhibitors in the LV homogenates of cocaine-treated rats (Fig. 3C). Diphenyleneiodonium, a flavoprotein inhibitor, caused a marked attenuation in the increases levels of chemiluminescence caused by NADPH (−70%). This increase was unaltered by oxyypurinol, an inhibitor of xanthine oxidase,
whereas NBT caused a 99% decrease in the signal, confirming $O_2^-$ as the measured radical.

### 3.1.5. Expression of NADPH oxidase subunits

At the same time as the NADPH oxidase assay, the relative mRNA levels of gp91phox and p22phox were measured using real-time quantitative RT-PCR. Levels of mRNA encoding the gp91phox and p22phox subunits were significantly increased by 80 and 90% respectively, in the myocardium of the 2-day cocaine-administered rats compared to controls (Fig. 2D).
3.2. Protocol 2: effect of antioxidants on cocaine-induced cardiac injury

3.2.1. Cardiac functional parameters and LV weights

On day 8, echocardiography assessments of the left ventricular function were processed and the results are shown in Fig. 3. There was no difference (i.e. in the absence of cocaine) in echocardiographic parameters between the placebo- and vitamin-treated groups. Compared with controls, cocaine significantly reduced the left ventricular fractional shortening and cardiac index by 23 and 30% \( (P<0.05) \), respectively. These altered parameters were normalized by administration of antioxidant vitamins.

Left ventricular end-diastolic (LVEDD) and end-systolic...
3.2.2. Antioxidant enzyme activities and lipid peroxidation

LV tissue homogenates from the same animals as used for echocardiographic evaluations on day 8 of cocaine treatment were used for biochemical measurements. The results are shown in Table 3.

Although GPX activity was not altered by cocaine, CAT and SOD were significantly decreased in the cocaine group by $-65$ and $-21\%$ ($P<0.05$), respectively. Administration of vitamins maintained antioxidant activities to values similar to controls. Eight days of cocaine administration led to a $48\%$ increase in lipid peroxidation compared to control (Table 3). Vitamin C and E pre-treatment significantly prevented this peroxide content in the cocaine + vitamins group.

3.2.3. NADPH oxidase

As shown in Fig. 4A, NADPH oxidase activity was strongly increased in the cocaine group compared with the control group. Vitamin C and E treatment significantly attenuated this increase.

Gp91phox mRNA increased significantly in the cocaine group compared with control animals and significantly decreased in the cocaine + vitamins group. In contrast, no differences were found in the expression of p22phox (Fig. 4C).

4. Discussion

This study, performed in a rat model, demonstrates for the first time the role of reactive oxygen species as a trigger of cardiac injury induced by cocaine.

The first part of this study shows an early increase in lipid peroxidation as evidenced by increased MDA content after short-term cocaine administration. This lipid peroxidation is accompanied by an increase in the activity of antioxidant enzymes, namely GPX, SOD and CAT. The change in these activities at an early time point may represent an adaptive response in order to limit oxidative stress. This implies that there is increased generation of reactive oxygen species, which, in turn, leads to the upregulation of antioxidant systems [31]. Furthermore, we observed the production of $\mathrm{O}_2^-$, which significantly increased in LV homogenates of cocaine-treated rats after incubation with NAPDH. We can exclude the contribution of xanthine oxidase because oxypurinol, a specific xanthine oxidase inhibitor, did not modify superoxide production. Moreover, the flavoprotein inhibitor diphenylene iodonium attenuated NAPDH-stimulated superoxide production. Further evidence for the involvement of an NADPH oxidase in cocaine-induced oxidative stress was demonstrated by the significant decreases in the expression of p22phox and gp91phox.
NADPH oxidase in heart dysfunction. Interestingly, in our study, this early up-regulation of NADPH oxidase occurs in the absence of sustained hemodynamic changes, which suggests that oxidative stress may in fact precede the myocardial dysfunction observed after chronic cocaine abuse.

In the second part of the study, we observed that an 8 day treatment with cocaine induces a significant left ventricular dysfunction as evidenced by a decrease in the fractional shortening and cardiac index, together with cardiac hypertrophy, as assessed by increases in LV weight and LV diameters. These observations are similar to those observed in previous animal studies after repeated cocaine administration [35,36] and in humans after long-term cocaine abuse [2].

In these conditions, we confirm the presence of sustained oxidative stress as evidenced by increased MDA content. Moreover, the defense mechanisms against reactive oxygen species, i.e. direct detoxification through the action of antioxidant enzymes such as SOD and CAT, are impaired. These results show that the early adaptation observed after short-term cocaine administration is not sustained for long. In parallel to these changes, the NADPH oxidase activity remained increased in the cocaine group, thus confirming its contribution to cocaine-induced cardiac dysfunction.

Finally, in our study, the effects of vitamin C and E treatment on heart function and oxidative stress in response to repeated cocaine administration were determined. Vitamin E, because of its lipid solubility, prevents lipid peroxidation in biologic membranes. In this process, α-tocopherol is oxidized to an inactive α-tocopheroxyl radical. Ascorbic acid is a water-soluble antioxidant. It potentiates the effects of α-tocopherol by regenerating Fig. 4. Effects of vitamin E and vitamin C treatment on cocaine-induced NADPH oxidase on day 8 of cocaine administration. (A) NADPH oxidase activity in LV homogenates measured by lucigenin chemiluminescence. (B) gp91phox mRNA expression and (C) p22phox mRNA expression quantified by real-time quantitative RT-PCR. Levels were standardized to the expression of GAPDH. *P<0.05 vs. control, #P<0.05 vs. cocaine group.

gp91phox and gp91phox, two essential components of phagocyte-type NADPH oxidase, were increased in the LV of cocaine-treated rats. However, unlike in phagocytic cells, this activity is constitutively active [32]. In vivo, cardiac mRNA expression of p22phox and gp91phox was reportedly increased in the infarcted sites after myocardial infarction in rats [16]. An increase in the NADPH oxidase-derived ROS and protein expression of some subunits (gp91phox and p67phox) has also been found to be associated with a defect in contractile function in cardiac hypertrophy [33]. More recently, Li et al. [34] showed that the NADPH oxidase expressed in cardiomyocytes is increased in a guinea pig model of cardiac hypertrophy. These previous data outline the important pathophysiological role of provided because the mRNA expression of p22phox and gp91phox, two essential components of phagocyte-type NADPH oxidase, were increased in the LV of cocaine-treated rats. However, unlike in phagocytic cells, this activity is constitutively active [32]. In vivo, cardiac mRNA expression of p22phox and gp91phox was reportedly increased in the infarcted sites after myocardial infarction in rats [16]. An increase in the NADPH oxidase-derived ROS and protein expression of some subunits (gp91phox and p67phox) has also been found to be associated with a defect in contractile function in cardiac hypertrophy [33]. More recently, Li et al. [34] showed that the NADPH oxidase expressed in cardiomyocytes is increased in a guinea pig model of cardiac hypertrophy. These previous data outline the important pathophysiological role of NADPH oxidase in heart dysfunction. Interestingly, in our study, this early up-regulation of NADPH oxidase occurs in the absence of sustained hemodynamic changes, which suggests that oxidative stress may in fact precede the myocardial dysfunction observed after chronic cocaine abuse.

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in modulating the pathogenesis of cocaine-induced myocardial dysfunction, but complementary experimental studies are necessary, in particular to determine the useful dose and duration of treatment that can be used in humans.

Another explanation for the beneficial role of antioxidant treatment in the present study is also an effect on enzymes that generate ROS, considering the decrease of NADPH oxidase activity. A similar response has also been reported in vitamin-treated salt-loaded stroke-prone spontaneously hypertensive rats [37]. As recently reviewed by Azzi et al. [40], α-tocopherol produces the inhibition of PKC activity, and therefore decreases the phosphorylation and translocation of the cytosolic factor p47phox. Consequently, NADPH oxidase assembly is impaired and superoxide production is decreased. However, the present study extends the role of vitamins to the regulation of NADPH oxidase expression at the transcriptional level. Indeed, we observed for the first time to our knowledge that, in the vitamin-treated group, mRNA transcripts encoding gp91phox are decreased.

Although they were not specifically investigated here, there is the possibility that different cellular types contribute to superoxide production. Further experiments are necessary to determine whether cells contribute to the up-regulation of NADPH oxidase in LV homogenates after short-term and chronic administration.

In conclusion, our experiments demonstrate that: (1) cocaine administration induces early ROS production which precedes the sustained LV dysfunction seen after repeated administration of the drug; (2) a phagocyte-like NADPH oxidase contributes to this production; and (3) cocaine-induced cardiac dysfunction is prevented by antioxidant treatment. These results demonstrate the central role of reactive oxygen species in the development and progression of cardiomyopathy after cocaine abuse.

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