

Tobacco Smoke Induces Ventricular Remodeling Associated with an Increase in NADPH Oxidase Activity

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Key Words

Oxidative stress • Tobacco smoke exposure • Cardiac remodeling

Abstract

Background: Recent studies have assessed the direct effects of smoking on cardiac remodeling and function. However, the mechanisms of these alterations remain unknown. The aim of this study was to investigate the role of cardiac NADPH oxidase and antioxidant enzyme system on ventricular remodeling induced by tobacco smoke. **Methods:** Male Wistar rats that weighed 200-230 g were divided into a control group (C) and an experimental group that was exposed to tobacco smoke for a period of two months (ETS). After the two-month exposure period, morphological, biochemical and functional analyses were performed. **Results:** The myocyte cross-sectional area and left ventricle end-diastolic

dimension was increased 16.2% and 33.7%, respectively, in the ETS group. The interstitial collagen volume fraction was also higher in ETS group compared to the controls. In addition to these morphological changes, the ejection fraction and fractional shortening were decreased in the ETS group. Importantly, these alterations were related to augmented heart oxidative stress, which was characterized by an increase in NADPH oxidase activity, increased levels of lipid hydroperoxide and depletion of antioxidant enzymes (e.g., catalase, superoxide dismutase and glutathione peroxidase). In addition, cardiac levels of IFN- γ , TNF- α and IL-10 were not different between the groups. **Conclusion:** Cardiac alterations that are induced by smoking are associated with increased NADPH oxidase activity, suggesting that this pathway plays a role in the ventricular remodeling induced by exposure to tobacco smoke.

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Introduction

Exposure to tobacco smoke is a major risk factor for coronary heart disease (CHD), which is currently the leading cause of preventable death. Smoking increases the risk of death from CHD at least two-fold, and increases the risk of stroke by approximately 50% [1, 2]. It has been estimated that 440,000 deaths occur annually in the United States due to smoking [3].

More than 4,720 chemical compounds, in addition to an estimated 10^{15} - 10^{17} free radicals, are present in cigarette smoke [4, 5]. These compounds can cause vascular and tissue inflammation by stimulating inflammatory cells to produce reactive oxygen species (ROS). Initially, ROS production was thought to be an uncontrollable process that was associated with a pathological disease or a process that occurs at high levels (micromolar scale) in phagocytes to kill bacteria. Recently, ROS generation on a smaller scale (nanomolar scale) has been characterized in many cell types as a controlled, agonist-triggered and enzyme-dependent physiological process [6]. Isoforms of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex (Nox family NADPH oxidases) are the main cellular and vascular source of ROS with purposes of compartment-specific redox signaling [5]. NADPH oxidases are highly regulated, transmembrane enzymes present in a variety of phagocytic and nonphagocytic cells in several tissues, such as the vascular endothelium and the heart. NADPH oxidases catalyze the transfer of electrons from NADPH to molecular oxygen, leading to superoxide anion (O_2^-) and other derived products [6]. There is increasing evidence that NADPH oxidase activity is the main source of ROS involved in noxious effects of cigarette smoke [7-9].

In addition to the well-known pathological effects of cigarette smoke on vascular systems, recent studies have also assessed the direct effects of smoking in cardiac remodeling and function. Such studies have shown that tobacco smoke induces left atrium and ventricle enlargement, myocyte hypertrophy and systolic dysfunction [10-14]. The potential mechanisms for these alterations include hemodynamic and neurohormonal changes [15], metalloproteinases [16] and mitogen-activated protein kinases activation [17]. These and other effects may be associated with oxidative stress [18, 19].

It is well known that oxidative stress affects the extracellular matrix, myocyte contractile proteins and

myocyte hypertrophy; this oxidative stress can ultimately cause myocyte death via necrosis or apoptosis. Animals that have been exposed to tobacco smoke undergo cardiac structural changes that can be observed with electron microscopy; interestingly, these changes can be alleviated with beta-carotene supplementation [18]. Rats exposed to cigarette smoke undergoing experimental myocardial infarction show decreased reduced/oxidized glutathione ratios in heart and liver tissue [19]. However, no studies have addressed whether NADPH oxidase participates in the cardiac remodeling induced by smoking.

The aim of this study is to investigate the role of cardiac NADPH oxidases and antioxidant enzyme systems on ventricular remodeling induced by tobacco smoke.

Materials and Methods

All experiments and procedures were performed in concordance with the National Institute of Health's Guide for the Care and Use of Laboratory Animals and were approved by the Animal Ethics Committee of our institution.

Male Wistar rats that weighed 200-230 g were divided into 2 experimental groups: a control group that was not exposed to cigarette smoke (group C; $n=10$), and a group that was exposed to cigarette smoke for 2 months (group ETS $n=10$). Food and water were supplied *ad libitum*. The rats were observed for a period of 60 days, during which morphological, biochemical and functional analyses were performed. The ETS rats were exposed to cigarette smoke in a chamber (dimensions 95x80x65 cm) that was connected to a smoking device based on a model published by Wang et al. [20] and adapted by Paiva et al. [21]. The smoke was drawn out of filtered commercial cigarettes (composition per unit: 1.1 mg nicotine, 14 mg tar and 15 mg carbon monoxide) with a vacuum pump and exhausted into the smoking chamber. During the first week, the number of cigarettes was gradually increased from 5 to 10 cigarettes over a 30 min period, administered twice each afternoon. From this point on, 10 cigarettes were used for each exposure, and the rats were exposed four times/day, twice in the morning and twice in the afternoon.

Echocardiographic analysis

After 2 months of exposure, all of the animals were weighed and evaluated with a transthoracic echocardiograph exam. The exams were performed using a commercially available echocardiograph (SONOS 2000, Hewlett-Packard Medical Systems, Andover, MA) that was equipped with a 7.5 MHz phased array transducer. Imaging was performed with a 60° sector angle and 3 cm imaging depth. The rats were lightly anesthetized with an intramuscular (IM) injection that was a mixture of ketamine (50 mg/kg) and xylazine (1 mg/kg). The rat chests were shaved, and the rats were placed in a

left lateral position. Targeted 2-D M-mode echocardiograms were obtained from short-axis views of the left ventricle (LV) at or just below the tip of the mitral-valve leaflets, as well as at the level of aortic valve and left atrium. M-mode images of the LV, left atrium and aorta were recorded on a black-and-white thermal printer (Sony Up-890MD) at a sweep speed of 100 mm/s. All of the tracings were manually measured with a caliper by the same observer using the leading-edge method recommended by the American Society of Echocardiography [22]. The data represent the mean of measurements from at least five consecutive cardiac cycles. The LV end-diastolic dimension (LVDD) and posterior wall thickness (LVWT) were measured at maximal diastolic dimension, whereas the end-systolic dimension (LVSD) was measured at maximal anterior motion of the posterior wall. The left atrium was measured at its maximal diameter, and the aorta was measured at the end of diastole. The LV systolic function was assessed by calculating the fractional shortening $\{(LVDD - LVSD)/LVDD \times 100\}$ and the ejection fraction $\{(LVDD^3 - LVSD^3)/LVDD^3\}$. The transmitral diastolic flow (E and A) velocities were obtained from the apical four-chamber view. The E/A ratio was used as an index of LV diastolic function.

Morphometric analysis

At the completion of the functional analyses, the right and left ventricles (including the interventricular septum) were dissected, separated and weighed. Transverse sections of the LV were fixed in 10% buffered formalin and embedded in paraffin. Five-micron-thick sections were stained with hematoxylin and eosin (HE) or the collagen-specific stain picrosirius red (Sirius red F3BA in aqueous saturated picric acid). The myocyte cross-sectional area was determined for a minimum of 100 myocytes per HE-stained cross section. The measurements were obtained from digital images (400x magnification) that were collected with a video camera that was attached to a Leica microscope; the images were analyzed with the Image-Pro Plus 3.0 software (Media Cybernetics; Silver Spring, MD). The myocyte cross-sectional area was measured with a digital pad, and the selected cells were transversely cut so that the nucleus was in the center of the myocyte [21]. The interstitial collagen volume fraction was determined for the entire cardiac section that was stained with picrosirius red by analyzing digital images that were captured under polarized light (200x magnification). The cardiac tissue components were identified according to the following staining patterns: red for collagen fibers, yellow for myocytes and white for interstitial space. The collagen volume fraction was calculated as the sum of all the connective tissue areas divided by the sum of all the connective tissue and myocyte areas. On average, 35 microscopic fields were analyzed per heart with a 20x lens. Perivascular collagen was excluded from this analysis [21].

Cardiac lipid hydroperoxide and antioxidant enzyme analysis

Left ventricle samples (200 mg) were homogenized in 5 mL of chilled phosphate buffer (0.1 M, pH 7.4) in a motor-driven Teflon glass Potter *Elvehjem*. The homogenate

was centrifuged at 12,520 x g for 15 min at 0°C, and supernatant was used for measure total protein as described previously [23], lipid hydroperoxide (LH) through hydroperoxide-mediated oxidation of Fe²⁺ as previously published [23], and antioxidants enzyme activities. Glutathione peroxidase (GSHPx, E.C.1.11.1.9), superoxide dismutase (SOD, E.C.1.15.1.1) and catalase (CAT, E.C.1.11.1.6) activity was assessed as previously specified [23-25]. The enzyme activity assays were performed at 25°C with a micro-plate reader (iQuant-MQX 200 with Kcjunior software connected to computer system control, Bio-Tec Instruments, Winooski, Vermont, USA). The absorbance was measured using a Pharmacia Biotech spectrophotometer (UV/visible Ultrospec 5000 with Swift II Applications software connected to computer system control, 974213, Cambridge, England, UK) at 560 nm. All of the reagents were from Sigma (St. Louis, Missouri, USA) [23].

NADPH oxidase activity in cardiac tissue

NADPH oxidase activity was assessed by HPLC analysis of dihydroethidium (DHE) oxidation products. Left ventricle samples (50 mg) were homogenized in lysis buffer (50 mM Tris, pH 7.4, 0.1 mM EDTA, 0.1 mM EGTA, 10 g/ml aprotinin, 10 g/ml leupeptin and 1 mM phenylmethylsulfonyl fluoride); samples were sonicated (3 cycles of 10 s each at 8 W) and centrifuged (18,000 g for 15 min) to separate the mitochondria and nuclei. The supernatants were further centrifuged at 100,000 g for 1 h to obtain a membrane-enriched fraction. HPLC analysis was subsequently used to measure the NADPH oxidase activity of such membrane fraction [6, 26-28].

The membrane fraction (20 µg protein) was incubated in PBS/DTPA with DHE (150 µM) and NADPH (300 µM) at a final volume of 120 µL at 37°C in the dark for 30 min. The reaction was stopped on ice and the homogenate centrifuged (5 min, 1,000 g) to yield a clear supernatant for HPLC injection [26]. One hundred microliters was injected onto the HPLC column. HPLC separation of DHE, 2-hydroxyethidium (EOH) and ethidium was performed as described previously with the specified modifications [26]. To identify possible molecules that could interfere with the DHE-derived fluorescence, we assessed both the EOH and ethidium products of DHE oxidation. While the optimal range of EOH detection is 570–580 nm (emission), 595 nm is the optimal emission wavelength for both hydroxyethidium and ethidium detection. Chromatographic separation was carried out on a NovaPak C18 column (3.9 x 150 mm, 5 µm particle size) in an HPLC system (Waters) that was equipped with a rheodyne injector, photodiode array (W2996) and fluorescence (W2475) detectors. Solutions A (pure acetonitrile) and B (water, 10% acetonitrile and 0.1% trifluoroacetic acid) were used as a mobile phase at a flow rate of 0.4 ml/min. The runs were started with 0% solution A, and the buffer was increased linearly to 40% solution A during the initial 10 min. The buffer composition was kept at this proportion for another 10 min, then changed to 100% for 5 min and then changed to 0% solution A for the final 10 min. DHE was monitored by ultraviolet absorption at 245 nm. EOH and ethidium were

monitored by fluorescence detection with an excitation wavelength of 510 nm and emission wavelength of 595 nm [6, 28].

NADPH oxidase activity was quantified by comparing the EOH integrated peak areas between the experimental and standard solutions under identical chromatographic conditions [27].

Evaluation of cardiac cytokine production

Cardiac cytokine production was performed as previously described [29-30]. Cytokine levels of IFN- γ , TNF- α and IL-10 in cardiac homogenate were evaluated by ELISA according to manufacturer instructions (R & D Systems, Minneapolis, MN, USA).

Statistical analysis

Statistical comparisons between the groups were performed with a Student's *t*-test for parameters with normal distribution. Otherwise, comparisons between groups were made with the Mann-Whitney U test. The data are expressed as the mean $\pm$ SD or the median (including the lower quartile and upper quartile). Data analysis was carried out with the SigmaStat software for Windows v2.03 (SPSS Inc, Chicago, IL). P-values less than 0.05 were considered to be statistically significant.

Results

The echocardiographic data are listed in Table 1. The ETS group had enlargement of the left chamber compared to the controls. The body weight (BW)-corrected left atrium (LA) and left ventricle end-diastolic dimension (LVDD) was increased 16.3% and 33.7%, respectively, in the ETS group. In addition to these morphological changes, the ejection fraction (EF) and fractional shortening (FS) were decreased in the ETS group. Rats that were exposed to tobacco smoke had approximately a 10% reduction in FS compared to the control rats ($C = 55.1 \pm 4.7\%$ and $ETS = 49.7 \pm 4.4\%$; $p = 0.016$).

The morphological data are listed in Table 2. The BW corrected left ventricular weight (LVW) was elevated in the ETS group, although this difference was not statistically significant. In contrast, the myocyte cross-sectional area (CSA), which is an index of ventricular hypertrophy, was increased 16.2% in the ETS group ($C = 303 \pm 28 \mu\text{m}^2$ and $ETS = 352 \pm 44 \mu\text{m}^2$; $p = 0.008$). The interstitial collagen volume fraction was also higher in ETS group compared to the controls ($C = 2.2\%$, range 1.9-2.8% and $ETS = 3.1\%$, range 2.6-3.6%; $p = 0.009$).

The data describing the cardiac lipid hydroperoxide and antioxidant enzyme activities are listed in Table 3.

	Group C n=10	Group ETS n=10	P-value
BW (g)	378 $\pm$ 44	343 $\pm$ 40	0.080
HR	357 $\pm$ 78	374 $\pm$ 56	0.582
LA (mm)	3.9 $\pm$ 0.6	4.1 $\pm$ 0.7	0.501
LA/BW (mm/kg)	10.4 $\pm$ 1.6	12.1 $\pm$ 1.6	0.029
LVEDD (mm)	7.0 $\pm$ 1.0	7.8 $\pm$ 0.5	0.036
LVEDD/BW (mm/kg)	18.9 $\pm$ 3.6	23.0 $\pm$ 1.8	0.005
LVESD (mm)	3.2 $\pm$ 0.7	3.9 $\pm$ 0.4	0.013
LVESD/BW (mm/kg)	8.6 $\pm$ 2.3	11.5 $\pm$ 1.3	0.003
LVWT/LVEDD	0.20 (0.17-0.22)	0.15 (0.14-0.18)	0.064
E/A	1.6 $\pm$ 0.4	1.6 $\pm$ 0.2	1.00
EF	0.91 $\pm$ 0.03	0.87 $\pm$ 0.03	0.008
FS (%)	55.1 $\pm$ 4.7	49.7 $\pm$ 4.4	0.016

Table 1. Echocardiographic data. C: control animals; ETS: animals exposed to tobacco smoke; BW: body weight; HR: heart rate; LA: left atrium; LV: left ventricle; LVEDD: LV end-diastolic dimension; LVESD: LV end-systolic dimension; LVWT: LV posterior wall thickness; EF: ejection fraction; FS: fractional shortening; E: peak velocity of early ventricular filling; A: peak velocity of transmitral flow during atrial contraction. The data are expressed as the mean $\pm$ SD or the median (including the lower quartile and upper quartile).

	Group C n=10	Group ETS n=10	P-value
BW (g)	378 $\pm$ 44	343 $\pm$ 40	0.079
LVW/BW (mg/g)	1.94 $\pm$ 0.11	2.07 $\pm$ 0.16	0.048
RVW/BW (mg/g)	0.52 (0.48-0.55)	0.58 (0.51-0.64)	0.251
CSA (μm^2)	303 $\pm$ 28	352 $\pm$ 44	0.008
IC (%)	2.2 (1.9-2.8)	3.1 (2.6-3.6)	0.009

Table 2. Morphological data. C: control animals; ETS: animals exposed to tobacco smoke; BW: body weight; LVW: left ventricular weight; RVW: right ventricular weight; CSA: cross-sectional area; IC: interstitial collagen volume fraction. The data are expressed as the mean $\pm$ SD or the median (including the lower quartile and upper quartile).

Lipid hydroperoxide was increased almost 2-fold in the ETS group compared to the controls ($C = 329.8$ nmol/g, range 320.8-366.8 nmol/g and $ETS = 629.3$ nmol/g, range 675.5-741.3 nmol/g; $p < 0.001$). In addition, cardiac antioxidant enzyme activity (e.g., catalase, superoxide dismutase and glutathione peroxidase) were lower in the ETS group compared to the controls.

Figure 1 shows the cardiac NADPH oxidase activity, which was increased in the ETS group ($n=6$) compared to the controls ($n=4$) ($C = 2.42$ mM/ μg

	Group C N=6	Group ETS N=8	P-value
LH (nmol/g tissue)	329.8 (320.8-366.8)	692.3 (675.5-741.3)	<0.001
CAT (μ mol/mg protein)	1.08 $\pm$ 0.18	0.56 $\pm$ 0.14	<0.001
GSH-Px (nmol/mg tissue)	37.84 $\pm$ 4.64	29.95 $\pm$ 4.59	0.008
SOD (nmol/mg of protein)	3.96 (3.83-4.11)	2.30 (1.96-2.95)	<0.001
TP (mg/100 mg tissue)	55.68 $\pm$ 2.16	46.82 $\pm$ 2.71	<0.001

Table 3. Total protein, lipid hydroperoxide concentration and antioxidant enzyme activity in the cardiac tissue. C: control animals; ETS: animals exposed to tobacco smoke; LH: lipid hydroperoxide; CAT: catalase; GSH-Px: glutathione peroxidase; SOD: superoxide dismutase; TP: total protein. The data are expressed as the mean $\pm$ SD or the median (including the lower quartile and upper quartile).

of protein, range 1.78-7.52 mM/ μ g and ETS = 37.02 mM/ μ g of protein, range 24.59-145.45 mM/ μ g; $p = 0.01$)

In addition, there were no differences of cardiac levels of IFN- γ (C = 6.89 (5.83-12.16) pg/ml and ETS = 7.70 (2.99-12.29) pg/ml; $p = 0.914$), TNF- α (C = 12.27 (11.44-35.63) pg/ml, ETS = 14.96 (12.46-20.98) pg/ml; $p = 0.762$) and IL-10 (C = 11.50 (11.09-19.61) pg/ml and ETS = 13.39 (8.90-19.19) pg/ml; $p = 0.914$) between the groups.

Discussion

Our study shows that tobacco smoke exposure induces morphological alterations and systolic dysfunction in rats. Importantly, these alterations were related to augmented heart oxidative stress, which was characterized by increased NADPH oxidase activity, increased levels of lipid hydroperoxide and depletion of antioxidant enzymes.

In accordance with previous reports, our data indicate that tobacco smoke exposure increases the size of the left cardiac chambers, induces myocardial hypertrophy and fibrosis and disrupts systolic function. Changes in the ventricular mass, volume and geometry are characteristic features of ventricular remodeling [31-33]. Although ventricular remodeling initially can be a compensatory process, ventricular remodeling eventually leads to progressive ventricular dysfunction, heart failure and sudden death [31-33].

The mechanisms involved in ventricular remodeling induced by tobacco smoke exposure remain poorly understood. However, a recent study has shown that smoking increases ROS production and oxidative stress, which may lead to vascular remodeling [34]. Overall cellular oxidative burden is regulated by a balance between the rates of ROS generation and a variety of

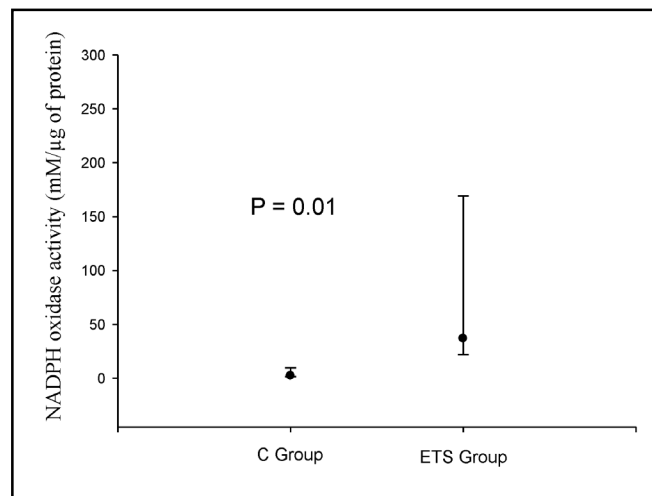


Fig. 1. The cardiac NADPH oxidase activity, which was increased in the EST group compared to the controls.

antioxidant enzymes/pathways, including catalase, superoxide dismutase, glutathione peroxidase, thioredoxin and small molecules such as vitamins. ROS are generally believed to be harmful because they cause oxidative damage to DNA, protein, lipids and other macromolecules [35]. At nanomolar concentrations, these free radicals may play an important role in physiological processes, for example, by functioning as a second messenger in signal transduction pathways. Thus, another, and perhaps the most significant, way excessive ROS can be deleterious is through disruption of signaling networks [6, 36].

In the vascular endothelium, it is well established that cigarette smoke induces ROS production via NADPH oxidase activation [7-9]. Previous studies also suggest that tobacco smoke may induce ventricular remodeling via changes in oxidative stress [18, 19]. However, the role that NADPH oxidase plays in cardiac remodeling that is induced by smoking is unknown.

In this study, we found that cigarette smoke exposure induces NADPH oxidase activation in the cardiac tissue. In addition, the cardiac antioxidant enzymes were depleted, and lipid hydroperoxide was increased in rats that were exposed to cigarette smoke. Our data reinforce the role of oxidative stress in cardiac damage induced by smoking; furthermore, the data suggest, for the first time, that NADPH oxidase plays a key role in the ventricular remodeling induced by tobacco smoke exposure.

NADPH oxidase activity was also increased in other models of ventricular remodeling and heart failure [37-39]. Two main isoforms are expressed in the heart - NADPH oxidase 2 and NADPH oxidase 4 [39], and experimental studies suggest that both isoforms may be involved in left ventricular hypertrophy. Additionally, NADPH oxidase 4 appears to be involved in pressure overload hypertrophy [37]. NADPH oxidase activity in left ventricular hypertrophy is modulated by mitogen-activated protein kinase (MAPK) activation. Interestingly, the MAPK pathway is also activated by tobacco exposure [17]. Thus, the myocyte hypertrophy that was present in the rats that were exposed to tobacco smoke may be associated with MAPK activation and increased NADPH oxidase activity.

In addition to the cardiomyocyte, other cell types present in cardiac tissue may have contributed to increased NADPH oxidase activity and ROS generation, such as endothelial and smooth muscle cells from microvessels, adventitial and interstitial fibroblasts and, particularly, infiltrating inflammatory cells. However, in this study there are no alterations in cardiac cytokine profile induced by cigarette smoke exposure, suggesting that inflammatory cells may not contribute to the increased NADPH oxidase activity seen in this model. Whether any of these cell types contributed

to increased ROS should be determined in future studies.

Cardiac fibrosis is a hallmark of deleterious cardiac remodeling. In the present study, tobacco smoke exposure increased the interstitial collagen volume fraction. Collagen accumulation is associated with conduction abnormalities and progressive cardiac dysfunction. Although the collagen increase in ETS group was not associated with increased mortality, it may have contributed to left ventricular dysfunction. There is strong evidence that NADPH oxidase is involved in the development of interstitial fibrosis [39, 40]. Indeed, in a pressure overload model, interstitial fibrosis was inhibited in NADPH oxidase 2-deficient mice, even though the extent of hypertrophy was similar to the controls [40]. Thus, this data suggest that different pathways modulate by NADPH oxidase activity are involved in fibrosis and hypertrophy.

Because NADPH oxidase plays a direct role in pathophysiological cardiac remodeling, tobacco-induced changes in NADPH oxidase activity may influence adverse ventricular remodeling via direct oxidative damage or by activating signal transduction pathways that cause myocyte hypertrophy, cardiac fibrosis and systolic dysfunction. Further studies are needed to elucidate the specific mechanism involved in NADPH oxidase mediated cardiac remodeling in this model.

In conclusion, cardiac alterations caused by smoking are associated with increased NADPH oxidase activity, suggesting that this enzymatic pathway plays a role in the ventricular remodeling induced by tobacco smoke exposure.

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