Original Article

Smoking is Associated with Remodeling of Gap Junction in the Rat Heart: Smoker's Paradox Explanation?

Rosangela Novo[1], Cristiana M. Freire[1], Sergio Felisbino[2], Marcos F. Minicucci, Paula S. Azevedo[1], Leonardo A. M. Zornoff[1], Sergio A. R. Paiva[1]

Faculdade de Medicina de Botucatu[1]; Instituto de Biociências de Botucatu[2], Botucatu, SP – Brazil

Abstract

Background: In a previous study utilizing the rat model, exposure to tobacco smoke for 5 weeks increased survival after AMI, despite similar age and infarct size between the smokers and nonsmokers, and absence of reperfusion.

Objective: Thus, this study aimed to analyze the effects of exposure to tobacco smoke on intensity, distribution or phosphorylation of connexin 43 in the rat heart.

Methods: Wistar rats weighing 100 g were randomly allocated into 2 groups: 1) Control (n = 25); 2) Exposed to tobacco smoke (ETS), n = 23. After 5 weeks, left ventricular morphometric analysis, immunohisthochemistry and western blotting for connexin 43 (Cx43) were performed.

Results: Collagen volume fraction, cross-sectional areas, and ventricular weight were not statistically different between control and ETS. ETS showed lower stain intensity of Cx43 at intercalated disks (Control: 2.32 ± 0.19; ETS: 1.73 ± 0.18; p = 0.04). The distribution of CX43 at intercalated disks did not differ between the groups (Control: 3.73 ± 0.12; ETS: 3.20 ± 0.17; p = 0.18). ETS rats showed higher levels of dephosphorylated form of Cx43 (Control: 0.45 ± 0.11; ETS: 0.90 ± 0.11; p = 0.03). On the other hand, total Cx43 did not differ between control and ETS groups (Control: 0.75 ± 0.19; ETS: 0.93 ± 0.27; p = 0.58).

Conclusion: Exposure to tobacco smoke resulted in cardiac gap junction remodeling, characterized by alterations in the quantity and phosphorylation of the Cx43, in rats hearts. This finding could explain the smoker's paradox observed in some studies (Arq Bras Cardiol. 2013;100(3):274-280).

Keywords: Smoking/physiopathology; Smoke Inhalation Injury/mortality; Arrhythmias,Cardiac; Rats.

Introduction

Despite convincing data on the harmful effects of smoking, several studies have reported low mortality after acute myocardial infarction (AMI) for smokers compared with nonsmokers. This phenomenon has been referred as the smoker's paradox [1-4].

Many explanations have been proposed for the more favorable outcomes in smokers after AMI including: 1) smokers are younger and present fewer co-morbidities; 2) they have higher pre-hospital mortality; 3) smokers have a larger thrombus burden, inducing a greater effectiveness of intravenous thrombolysis; 4) ischemic preconditioning-like effect is induced by tobacco smoke. Ischemic preconditioning is a phenomenon whereby a brief period of ischemia renders the myocardium resistant to infarction from a subsequent ischemic insult. Ischemic preconditioning limits infarct size, reduces the incidence of fatal arrhythmias, and protects against stunning myocardium [5,6].

In a previous study using the rat model, exposure to tobacco smoke for 5 weeks increased survival after AMI, despite similar age and infarct size between the smokers and nonsmokers, and absence of reperfusion. Importantly, the reduction of mortality was observed within the initial 24 hours after coronary occlusion [7]. In this model, 87% of early deaths were a consequence of ventricular arrhythmias [8]. Therefore, we assumed that the smoker's paradox in this model could be a result of a preconditioning-like effect. However, the potential mechanisms of this protection induced by exposure to tobacco smoke are still unknown.

Ischemic preconditioning protection depends on functional channels of gap junction intercellular communication [9]. The gap junctions in myocardium are specialized intercellular contacts that allow electrical impulse propagation among cardiomyocytes. Connexin 43 (Cx43) is the principal gap junction protein in ventricles of the heart [10] and plays a major role in ischemic preconditioning. This fact is evidenced by

the finding that ischemic preconditioning cannot induce protection in heterozygous Cx43-deficient mice[11]. However, the effects of smoking on cardiac Cx-43 remain unknown[12]. Therefore, to address this issue, we tested the hypothesis that exposure to tobacco smoke would interfere with the intensity, distribution or phosphorylation of Cx43 in the rat heart.

Materials and Methods

Groups and Treatment

All experiments and procedures were performed in accordance with NIH guidelines for the Care and Use of Laboratory Animals and were approved by the Animal Ethics Committee of our Institution (protocol number 399).

Male Wistar rats weighing 100 g, were randomly allocated into 2 groups: 1) Control group (n = 25), control rats without exposure to tobacco smoke; 2) Group exposed to tobacco smoke (ETS), n = 23. The tobacco smoke exposure lasted for 5 weeks.

The ETS rats were exposed to cigarette smoke in a chamber (dimensions 95 x 80 x 65 cm) connected to a smoking device based on a model adapted by Paiva et al[7,13-17]. The smoke was drawn out of filtered commercial cigarettes (composition per unit: 1.1 mg of nicotine; 14 mg of tar; and 15 mg of carbon monoxide) with a vacuum pump and was exhausted into the smoking chamber. During wk 1, the number of cigarettes was gradually increased from 5 to 10 over a 30-min period, twice in the afternoon. For the next approximately 3–4 wk, until the rats reached the target weight, 10 cigarettes were used in each smoking trial, repeated 4 times/d, twice in the morning and twice in the afternoon. In a previous study, with similar tobacco exposure time, the carboxyhemoglobin levels in smoke-exposed rats were greater than in the control group (control rats: 0.9 ± 0.7% and ETS rats: 5.3 ± 2.8%, p = 0.008)[14]. Thus, the latter study confirmed the efficacy of the exposure of smoking animals to cigarette smoke. All animals were fed the same amount of diet, which was based on the food ingestion of the group ETS. After this period of tobacco smoke exposure, rats were euthanized by an excessive dosage of anesthesia (sodium pentobarbital 100 mg/kg of body weight). Rats in the ETS group were not exposed to tobacco smoke during the 12 h before euthanasia.

Morphometric Analysis

At the completion of the treatment period, the right and left ventricles (including the interventricular septum) were dissected, separated, and weighed. Coronal sections, approximately 3 mm in thickness, taken 5 mm from the tip of the ventricles were fixed in a 10% buffered formaldehyde solution for 48 hours followed by immersion in a 70% alcohol solution for 48 hours. After fixation, the tissues were embedded in paraffin. Histological sections four and six microns in thickness were stained, respectively, with Hematoxylin-Eosin (HE) and collagen-specific Picro Sirius red. The myocyte cross-sectional areas (MA) were measured in the HE-stained sections by a LEICA DM LS microscope connected to a video camera and IBM compatible computer, equipped with the image analysis program Image Pro-plus (Media

Cybernetics, Silver Spring, Maryland, USA). Fifty to seventy cells were analyzed per slide. The selected cells presented a circular shape and were located in the subendocardial layer of the ventricular walls. This precaution was taken in order to optimize myocyte shape uniformity among the groups. The average cross-sectional area obtained for each group was used as an indicator of cell hypertrophy. The slides stained with Picro Sirius red were used to quantify the interstitial collagen volume fraction using video densitometry. Images of the cardiac tissue were taken and analyzed using the system described above. The elements of the cardiac tissue were identified according to color level. Therefore, the collagen fibers were visualized in red and the myocytes in yellow. The collagen volume fraction (CVF) was calculated automatically and corresponded to the sum of the collagen areas divided by the sum of the collagen tissue and myocyte areas. An average of thirty fields was analyzed using a lens with 40X magnification. Perivascular collagen was excluded from the analysis[14,16].

Immunohistochemistry for connexin 43

Immunohistochemistry experiments were performed according to Saffitz et al[18] with some modifications[19]. All hearts, transversally cut, were fixed, successively, in 10% buffered formaldehyde solution for 24 hours, running water for 24 hours and 70 percent alcohol for 24 hours. After fixation, tissues were embedded in paraffin and sliced into 3-μm sections. Before immunolabeling, tissue characterization and orientation were recorded by hematoxylin-eosin staining.

The sections (3 μm) were placed on poly-D-lysine-coated glass slides and successively dewaxed three times, for 5 minutes each, in baths of xylol, 100% alcohol, 95% alcohol and 70% alcohol followed by rehydration in distilled water for 10 minutes. Antigenic sites were retrieved by treatment of slices in 0.01 M acid citric buffer in phosphate-saline buffer (PBS) pH 6.0 for 10 minutes in microwave environment. After this period, the slices were kept ice-cold for 20 minutes followed by washing in PBS pH 7.2. The sections were blocked with 3% serum albumin bovine (BSA) solution in PBS for 30 minutes at room temperature. After this procedure, the sections were incubated overnight with rabbit polyclonal anti-connexin 43 antibody (Abcam Inc. Cambridge) diluted 1:100 in 2% BSA in PBS at 4 °C. On the next morning, the sections were washed in PBS for 15 minutes three times and incubated in secondary antibody (Texas-red anti-rabbit IgG, Vector) at 1:50 dilution in 2% BSA in PBS at room temperature for 1 hour.

The sections were washed in PBS for 15 minutes three times and mounted in glycerol medium. Ten sections were processed simultaneously in each immunolabeling run while negative controls, in which the primary antibody was omitted, were routinely run in parallel[20] with the objective of monitoring any occurrence of unspecific stain. Immunolabeling of Cx43 was examined by epi-fluorescent microscopy (Carl-Zeiss, Inc. North America) equipped with a WG filter cube (excitation = 550 nm and emission = 650 nm) connected to a video camera and compatible computer (AxioPlan 4.1; Carl-Zeiss Inc.). All images were recorded on the same day as labeling and the tissues were coded and examined blindly by the same person.

Original Article

From each heart, as described by Kostin et al[21], 10 optical fields were recorded and analyzed for distribution of Cx43 in the myocytes (i.e. presence of Cx43 within or outside the intercalated disk) and intensity of Cx43 staining at intercalated disks.

Western blotting for connexin 43

The western blotting experiments were performed according to Rolim et al[22]. The samples were homogenized in ice-cold lyses buffer (50 mM potassium phosphate buffer pH 7.0; 0.3 mM sucrose ; 0.5 mM DTT; 1 mM EDTA pH 8.0; 0.3 mM PMSF; 10 mM NaF and protease inhibitor cocktail).

The supernatant was collected by centrifugation at 12000 rpm for 20 minutes and stored at -80 °C. The determination of proteins was performed by BSA protein standard (Bio-Rad, Hercules, CA, USA) curves and the Bradford method. The protein samples were diluted in Laemmli buffer (240 mM Tris-HCl; 0.8% SDS; 40% glycerol; 0.02% bromophenol blue and 200 mM β-mercapthoethanol). Then 50 μg of each sample was separated on 10% polyacrylamide stacking gels (240 mM Tris-HCl pH 6.8; 30% polyacrylamide; APS and TEMED) and resolving gels (240 mM Tris-HCl pH 8.8; 30% polyacrylamide; APS and TEMED). Electrophoresis was run at 120 V for 90 minutes.

The transfer onto nitrocellulose membrane (Bio-Rad) was performed in the Mini-Trans Blot system (Bio-Rad, Hercules, CA, USA) with transfer buffer (25 mM Tris; 192 mM glycine; 20% methanol and 0.1% SDS) for 90 minutes at 120 mA. The blots were blocked with 5% non-fat milk for 2 hours at room temperature with shaking and washing three times in PBS Tween (1mM Tris pH 2.8; 5 mM NaCl and Tween 20). Afterwards, the blots were immunolabeled overnight with a monoclonal mouse anti-Cx43 primary antibody (Ab11369 - Abcam), corresponding to amino acids 130 – 143 of Cx43 diluted to 1: 250 in PBS Tween (1mM Tris pH 2.8; 5 mM NaCl and Tween 20) or monoclonal mouse anti- Cx43 (13-8300 – Zymed) diluted to 3μg/mL, specific to serine 368, an unphosphorylated residue of Cx43.

On the next morning, the membranes were washed three times in PBS Tween (1mM Tris pH 2.8; 5 mM NaCl and Tween 20). This was followed by incubation for 2 hours at room temperature with an HRP- conjugated anti-mouse antibody (Sigma) diluted to 1:10000. After rinsing three times in PBS Tween (1mM Tris pH 2.8; 5 mom NaCl and Tween 20), the membranes were incubated for 1 minute in ECL solution (Amershan) (*Solution I*: 2.5mM luminol, 400 μM p-cumaric acid, 100 μM tris pH 8.5; *solution II*: 5.4 mM 30% hydrogen peroxide, 100 μM tris pH 8.5). After this period, the membranes were exposed to x-ray film (Eastman Kodak Co, USA) for 10 minutes in a darkroom and then successively dipped in revealing solutions, water and fixative for 1 minute each. The reaction between ECL reagent and the HRP- conjugated anti-mouse antibody on the membranes emits light that appears as dark bands on the x-ray film after revelation.

After this procedure, densities of the dark bands obtained were analyzed by densitometric quantification of signal intensity by Scion Image (Scion Corporation, Frederick, Maryland, USA) software with the background measurements of signal intensity being subtracted individually from each Lane[19].

Statistical analysis

Data were expressed as means $\pm$ SD. The statistical analysis was performed by Student's *t* test. Differences were considered significant at level of 5%. Statistical analyses were performed using SigmaStat for Windows v2.03 (SPSS Inc, Chicago, IL).

Results

The morphometric variables did not differ statistically between control and ETS groups (Table 1).

Smoke exposure induced lower stain intensity of Cx43 at ID when compared to control rats (Control: 2.32 $\pm$ 0.19; ETS: 1.73 $\pm$ 0.18; p = 0.04). The distribution of Cx43 at ID did not differ between control and ETS rats (Control: 3.73 $\pm$ 0.12; ETS: 3.20 $\pm$ 0.17; p = 0.18); (Figure 1).

ETS rats showed higher values of dephosphorylated form of Cx43 than control rats (Control: 0.45 $\pm$ 0.11; ETS: 0.90 $\pm$ 0.11; p = 0.03). On the other hand, total Cx43 did not differ between control and ETS groups (Control: 0.75 $\pm$ 0.19; ETS: 0.93 $\pm$ 0.27; p = 0.58); (Figure 2).

Table 1 - Morphometric data

	Control (n = 25)	ETS (n = 23)	p
BW (g)	237 ± 20.5	230 ± 19.6	0.30
LV (g)	0.52 ± 0.05	0.52 ± 0.05	0.97
RV (g)	0.15 ± 0.015	0.15 ± 0.014	0.45
LV/BW (g)	2.21 ± 0.15	2.27 ± 0.14	0.15
RV/BW (g)	0.64 ± 0.05	0.67 ± 0.10	0.11
Atrium (g)	0.05 ± 0.015	0.045 ± 0.014	0.16
Myocyte area (μ^2)	190 ± 51.6	184 ± 30.8	0.68
Collagen (%)	4.34 ± 2.75	4.41 ± 0.96	0.90

Values are mean ± SD. n: number of rats; BW: body weight; LV: left ventricle; RV: right ventricle; LV/BW: left ventricle normalized by BW; RV/BW: right ventricle normalized by BW. Student's t test.
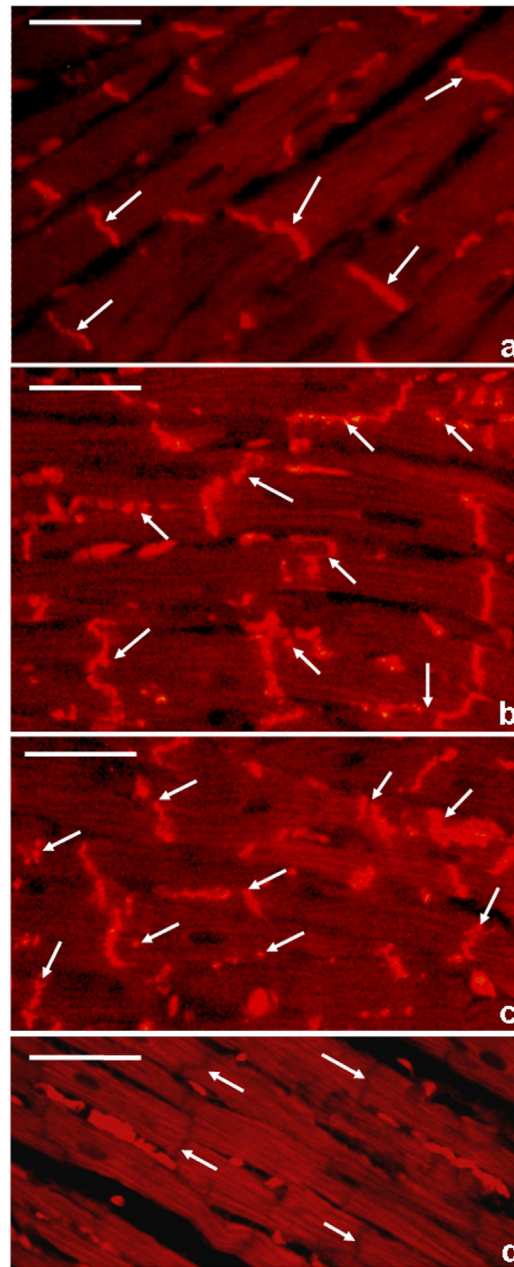
Figure 1 - *Cx43 immunofluorescence micrographs from sections of rat myocardium: control (A), exposed to tobacco smoke (B and C) and negative control (D). Arrows in A: Intercalated disks with immunoreactive signal for Cx43; in B and C: immunoreactive signal for Cx43 at intercalated disks and plasma membrane; in D: intercalated disks free of stain for Cx43. Bar = 20 µm.*

Discussion

This study aimed to analyze the effects of exposure to tobacco smoke on the intensity, distribution, and phosphorylation of Cx43 in the rat heart. Our data indicated that tobacco smoke exposure induced remodeling of the cardiac gap junctions.

An important issue to be considered is that, in addition to the already well-known effects on the vascular endothelium

and lungs, previous studies showed that cigarette smoke exposure that lasts at least two months induces cardiac remodeling, characterized by morphological and functional alterations. Indeed, smoking produced augmentations in the left ventricular diastolic and systolic diameters, and induced left ventricular hypertrophy. These morphological alterations were associated with reduction of left ventricular systolic function[7,13,14,16,19,23-27]. However, the effects of tobacco smoke exposure on the cardiac gap junctions are still unknown.
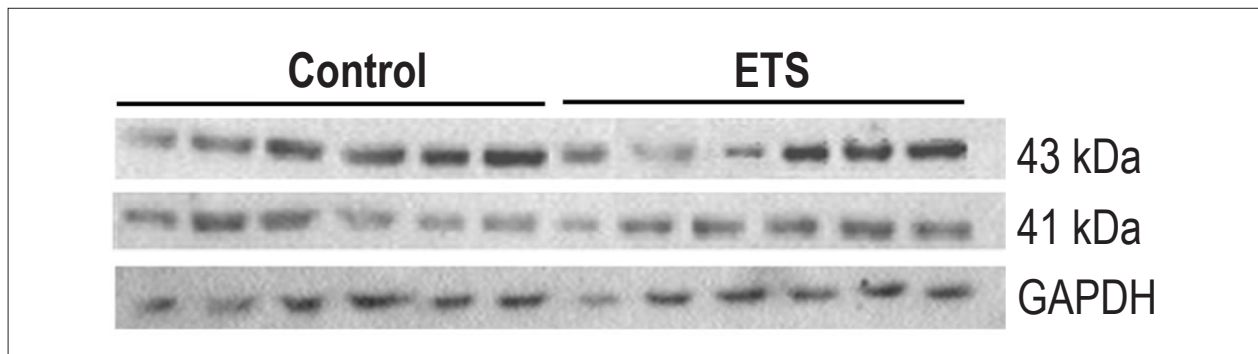
Figure 2 - *Western blot analysis. A: Blot with anti-total-Cx43, B: non-phosphorylated form of Cx43 detected by monoclonal mouse anti-np-Cx43. C: GAPDH bands.*

The main finding of the present study was that exposure to tobacco smoke induced remodeling of the cardiac gap junctions. Indeed, the remodeling process was characterized by a lower quantity of Cx43 at ID and an increase in the non phosphorylated form of Cx43 in the myocardium.

Another important issue is that cardiac remodeling characteristically starts with genetic, molecular, cellular, biochemical, and then structural alterations[28-31]. In this context, our findings of Cx43 alterations and no differences in the left and right ventricular morphological variables can be interpreted as the observation of an early stage of the cardiac remodeling. Therefore, we assume that Cx43 remodeling precedes the morphological and functional alterations induced by tobacco smoke exposure.

Gap junctions are clusters of transmembrane channels that connect the cytoplasmic compartments of neighboring cells. The component proteins of gap junction channels are termed connexins. There are many types of connexins, but the predominant isoform in the heart, mainly in the ventricle, is Cx43[10]. A major role of gap junctions in the myocardium is to coordinate electrical excitation and to facilitate intercellular change of small proteins, such as signal transduction proteins. Importantly, the normal gap junction functions are related to normal expression and distribution of Cx43, given that Cx43 is located mainly in the intercalated disc of cardiomyocytes. In addition, the degree of Cx43 phosphorylation is critical to gap junction function[10].

Alterations in the quantity, localization and phosphorylation of Cx43 have been observed when the heart was submitted to ischemic or hypoxic events[21,32,33] and when these events are preceded by ischemic preconditioning[34,35]. Such ischemia induces reduction of cardiac gap junction, characterized by lower expression of Cx43[32,36]. Thus, the observed alterations in Cx43 intensity provoked by smoking in the present study are similar to those induced by ischemia.

The diminished Cx43 staining intensity at ID induced by exposure to tobacco smoke in the present study resembles the effects of ischemic preconditioning, which initiates a process of Cx43 occurrence in the free plasma membrane, outside of the intercalated disks. As a consequence of this effect, ischemic preconditioning may improve the cell's chance of survival following prolonged ischemia[35].

With respect to the increase in the non-phosphorylated form of Cx43 induced by exposure to tobacco smoke, many studies have evidenced that the dephosphorylation of Cx43 causes electrical uncoupling in the cardiac myocytes[34] and the inhibition of Cx43 dephosphorylation lead to protective effects on cardiac function during hypoxic injury[37]. In this context, studies found that gap junction-uncoupling agents administered prior to ischemia preserve the electrical coupling of cardiac myocytes during subsequent ischemic insult, similarly to ischemic preconditioning[38]. These authors suggest that the partial and reversible uncoupling of gap junctions prior to ischemia results in an antiarrhythmic effect during a prolonged ischemic insult, similarly to ischemic preconditioning.

In this context, the present study found that exposure to tobacco smoke produced a remodeling process in the cardiac gap junction, an effect similar to ischemic preconditioning. Therefore, our data strongly suggest that exposure to tobacco smoke may induce an ischemic preconditioning-like effect in the rat heart. Importantly, this phenomenon could explain the smoker's paradox observed in some studies[1-4].

The mechanisms involved in the effects of tobacco smoke exposure on cardiac gap junctions are unknown. Among the possible mechanisms, ventricular remodeling induced by such exposure is associated with augmented activation of mitogen-activated protein kinases[23]. In addition, there is evidence that the PKC p38 and p42/44 MAPK pathways regulate Cx43 expression[39].

Our study should be analyzed considering a potential limitation. We did not include a preconditioning group. However, the alterations in the Cx43 induced by the preconditioning are well established. Therefore we strongly believe that our data add important information about this subject.

Conclusion

This study has shown that exposure to tobacco smoke resulted in cardiac gap junction remodeling, characterized by alterations in the quantity and phosphorylation of Cx43, in rats hearts. This finding could explain the smoker's paradox observed in some studies.

Novo et al.
Smoking and connexin 43 in the rat heart

Original Article

Author contributions

Potential Conflict of Interest

Sources of Funding

Study Association

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