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Coronary vasodilation impairment in pilocarpine model of epilepsy

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ABSTRACT

We investigated the coronary arteries reactivity alterations in rats with epilepsy induced by pilocarpine. To do so, male Wistar rats weighing between 250 g and 300 g were used. *Status epilepticus* (SE) was induced in rats using 385 mg/kg (i.p.) of pilocarpine. After 60 days from the first spontaneous seizure, rats were submitted to heart rate measurements and then, one day after, euthanized, and the heart was dissected and submitted to constant flow Langendorff approaches to evaluate coronary reactivity. Rats with epilepsy showed higher resting heart rate and impairment of coronary vasodilation induced by bradykinin. Endothelial nitric oxide synthase (eNOS) and superoxide dismutase (SOD) presented a reduced staining in coronary arteries, and eNOS expression was also reduced in the left ventricle of rats with epilepsy. Our findings demonstrated, for the first time, that epilepsy can cause impairment of coronary arteries reactivity, probably because of an endothelial dependent mechanism.

1. Introduction

After stroke, sudden unexpected death in epilepsy (SUDEP) is the second main cause of lost years of life [1]. In fact, SUDEP typically affects 1 in 4500 children and 1 in 1000 adults with epilepsy per year [2]. Many aspects need to be clarified about the exact mechanism underlying SUDEP, which appears to be multifactorial. It has long been demonstrated that seizures are accompanied by a series of changes in cardiovascular parameters [3]. Moreover, experimental and clinical studies show that these changes are not restricted to ictal periods [3,4]. Changes in heart rate (HR) are the main cardiovascular modification observed during seizures. Furthermore, higher resting HR has been shown both in patients and in experimental models of epilepsy [3–5]. It is worthy of note that a variation in HR, especially increased resting HR, is a risk factor for the development of cardiovascular diseases, such as atherosclerosis. Also, HR increase has been related to sheer stress and vascular endothelial injury [6]. Based on this background, the aim of our study was to evaluate the coronary arteries reactivity of rats with epilepsy induced by pilocarpine.

2. Materials and methods

2.1. Animals

Our study was performed with male Wistar rats (250–300 g) and was approved by the Institutional Ethics Committee of the Federal University of Goiás (CEUA 024/2012). All animals had access to water and rat chow ad libitum.

2.2. Induction of epilepsy

To reduce the peripheral effects of pilocarpine, adult male Wistar rats (250-300 g) received scopolamine methylnitrate (1 mg/kg, i.p.; Sigma, St. Louis, MO, US), followed 30 min later by administration of pilocarpine hydrochloride (385 mg/kg, i.p.; Sigma) to induce *status epilepticus* (SE). Seizure activity was monitored behaviorally and terminated with an injection of diazepam (10 mg/kg, i.p.; Santisa) after 4 h of SE. After a silent period, the rats began to present spontaneous recurrent seizures (SRS), lasting throughout the rat's life [7]. Only animals that displayed spontaneous seizures were included in this study. Experimental protocols were performed 60 days after the first spontaneous seizure (24 h per day by video monitoring). Control rats were submitted to the same protocol but received saline instead of pilocarpine.

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2.3. Heart rate and coronary reactivity protocol

After 60 days of SRS, rats were anesthetized with ketamin (100 mg/kg, i.p.) and xylazine (20 mg/kg, i.p.). For HR measurement, electrocardiogram (ECG) stainless steel electrodes were implanted just caudal of the diaphragm and in the mediastinum, as described by Sgoifo and coworkers [8]. Electrodes were subcutaneously conduced and exteriorized between scapulae. After 24 h of surgery recovery, ECG was recorded for 1 h. The ECG signals were amplified, filtered (0.5–100 Hz, model 12C 16OS, Technologies, Quincy, Massachusetts, U.S.A.) and sampled at 1000 Hz (Power Lab/8SP; AD Instruments, Melbourne, Australia). Then, the rats were euthanized by decapitation; the heart was carefully dissected and the connective tissue was removed. The heart was perfused through the aortic stump with Krebs-Ringer solution, pH 7.4 at 37 °C under 95% O_2, 5% CO_2, and at constant flow (8–10 ml/min). Coronary perfusion was measured with a pressure transducer that was connected to aortic cannula and coupled to a data-acquisition system (DATAQ Instruments, Akron, OH, US). After a basal period (30–40 min), the hearts from control or rats with epilepsy were randomly submitted to increasing concentrations (in bolus) of bradykinin (BK) (10^{-8} to 10^{-5}M), an endothelium-dependent vasodilator whose mechanism involves NO production. To evaluate the endothelium-independent vasorelaxation, sodium nitroprusside (SNP) (10^{-6} to 10^{-3}M), a NO donor, was injected into the perfusion system. A 10-min period of washout was realized between drug administration.

2.4. Immunohistochemistry staining levels

After ex vivo protocol the hearts were collected, the left ventricle (LV) was dissected, and the medial part of the LV was separated for histological analysis. The remaining tissue was immediately frozen in liquid nitrogen and stored in ultra-freezer (−80 °C) for Western Blot assay. The LV samples were fixed in 4% paraformaldehyde, embedded in paraffin and sectioned (5 μm). Sections were deparaffinized and rehydrated, and then underwent microwave antigen retrieval with sodium citrate (10 mM). Immunohistochemistry was performed using endothelial nitric oxide synthase (eNOS) (49G3; Cell Signaling Technology, Danvers, MA, US) and superoxide dismutase (SOD)-1 (FL-154; Santa Cruz Biotechnology, INC); both eNOS and SOD-1 were diluted 1:100 in BSA 1% (Cell Signaling Technology, Danvers, MA, US). Slices were incubated overnight at 4 °C. The bound antibody was visualized using the Novolink Polymer Detection System (Leica Biosystems, Wetzlar, GE) by following the manufacturer's instructions.

All images were taken at the same light intensity using a light microscope (Olympus BX43) at ×40 magnification. The immunostaining was quantified in different coronary arteries by the Image J analysis program. The areas of positive staining were identified using segmentation for 24-bit RGB images. The histogram-based segmentation for each channel was set up as follows: red: 0–164, green: 0–150, and blue: 0–150 pixel intensity. Percent staining was calculated from the data for positive staining and total area. Microscopy settings (light power) were determined at the beginning of each imaging session and then held constant during the analysis. Positive staining was compared by Student's *t*-test.

2.5. Western blot analysis

Samples of left ventricles were collected and homogenized in lysis buffer containing Phosphate - buffered saline (PBS), 1% Nonidet P40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 0.01% protease inhibitor cocktail (Na_3VO_4 2 mM, PMSF 10 μg/ml, leupeptin 1 μg/ml, aprotinin 1 μg/ml, pepstatin 1 μg/ml). Forty micrograms of protein from each sample were separated by polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Nonspecific binding was blocked by incubation in 5% nonfat milk and 0.1% Tween 20 in Tris-buffered saline. Membranes were probed with specific primary antibodies — eNOS (1:1000, Cell Signaling Technology, Danvers, MA) or SOD-1 (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA) — and exposed to chemiluminescence with ECL Western Blotting Detection Reagent kit (GE Healthcare Life Sciences). The blot was visualized on ImageQuant LAS 500 (GE Healthcare Life Sciences). Immunoreactive bands were quantified by Image J software using GAPDH (1:2000, Santa Cruz Biotechnology, Santa Cruz, CA) as a normalization control. The results were compared by Student's *t*-test.

3. Results

Our results showed higher resting HR for rats with epilepsy compared with that for control rats (Fig. 1A). Also, coronary BK-induced vasodilation was reduced in the epilepsy group, in ex vivo condition (Fig. 1B), whereas no difference in coronary SNP-induced vasodilation was found between groups (Fig. 1C). Furthermore, the SOD-1 (Fig. 2A, B) and eNOS (Fig. 2D, E) immunostaining was reduced in the coronary of rats with epilepsy. Furthermore, eNOS expression (Fig. 2F) was reduced in the left ventricle of rats with epilepsy. Differently, no differences were observed regarding the cardiac SOD-1 expression (Fig. 2C).

4. Discussion

First of all, our results are in agreement with our previous study [9] that shows that rats submitted to pilocarpine model of epilepsy have higher resting HR than control rats. Also, we showed that rats with epilepsy presented reduced BK-induced coronary vasodilation compared to control rats without differences when the vasodilation was induced by SNP. In addition, immunostaining analysis showed a lower staining area of both SOD and eNOS in the coronary endothelium of rats with epilepsy. Furthermore, we measured the protein expression of both SOD-1 and eNOS in the hearts. Our results showed a decreased eNOS expression in the left ventricle of rats with epilepsy with no changes of SOD-1 expression.

It is well-known that the endothelium plays a key role in the control of vascular tone, and changes of its function may lead to several disorders, such as hypertension and myocardial ischemia [10]. Nitric oxide is a potent vasodilator released by the endothelium; NO is produced by three isoforms of NO synthase, of which endothelial isoform (eNOS) is the most important isoform acting on the vascular bed [10]. It has been well-demonstrated that eNOS is involved in several aspects of vascular function ranging from control of blood pressure to antiatherosclerotic effects [11]. Therefore, eNOS is fundamental to guarantee the syntheses and NO bioavailability [10,12]. The NO oxidation to nitrite or nitrate, which is both vascular inactive nitrogen oxides, is another important factor to be observed concerning NO bioavailability [12]. In fact, it is well-known that reactive oxygen species (ROS), such as superoxide anion (O_2^-), reacts in a very fast manner with NO [12, 13]. In this scenario, antioxidant agents, like SOD, play a key role in the bioavailability of NO against the action of ROS, especially O_2^- [12]. Again, our results showed reduced coronary anti-SOD-1 staining, which was not confirmed by protein quantification in left ventricle. Even that, we believe that further experiments regarding SOD-1 activity and protein quantification in isolated endothelial cells would be interesting to clarify our understanding.

Hormones, inflammatory factors, ROS, catecholamines, and blood flow patterns are elements that can lead to impairment of vascular endothelial activity [14]. In this sense, shear stress plays an important role in vascular endothelial dysfunction. It is known that endothelial cells, when subjected to frequent flow variations and different shear stress levels tend to modify their gene expression, increasing the production of proinflammatory factors and ROS. Also, these responses are quite dependent on the frequency of flow variation [15].

Custodis et al. [16] showed that the reduction of HR in apolipoprotein E–deficient mice treated with ivabradine improved endothelial

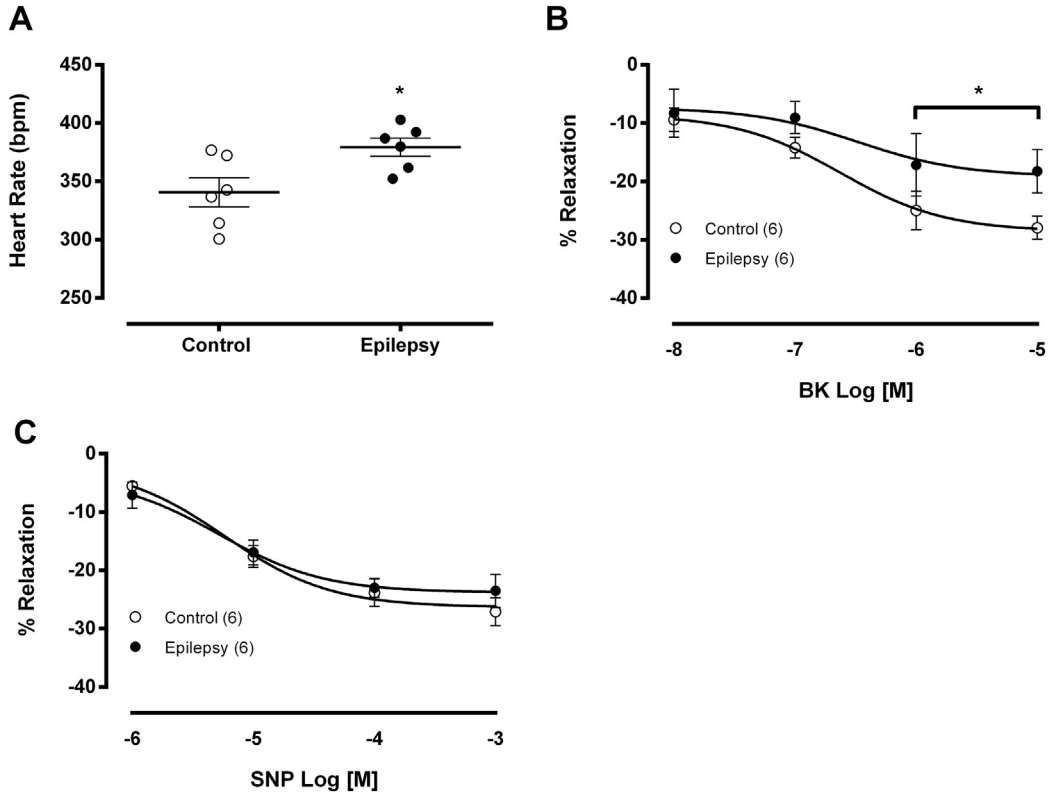

Fig. 1. (A) Mean resting HR of control and epilepsy groups. Concentration-response curves to bradykinin (B) and to sodium nitroprusside (C) in coronary of isolated heart of rats with epilepsy (closed circle) and control rats (open circle). *$p < 0.05$ compared to control group. The number of rats used is indicated in parentheses. BK: Bradykinin. SNP: Sodium Nitroprusside.

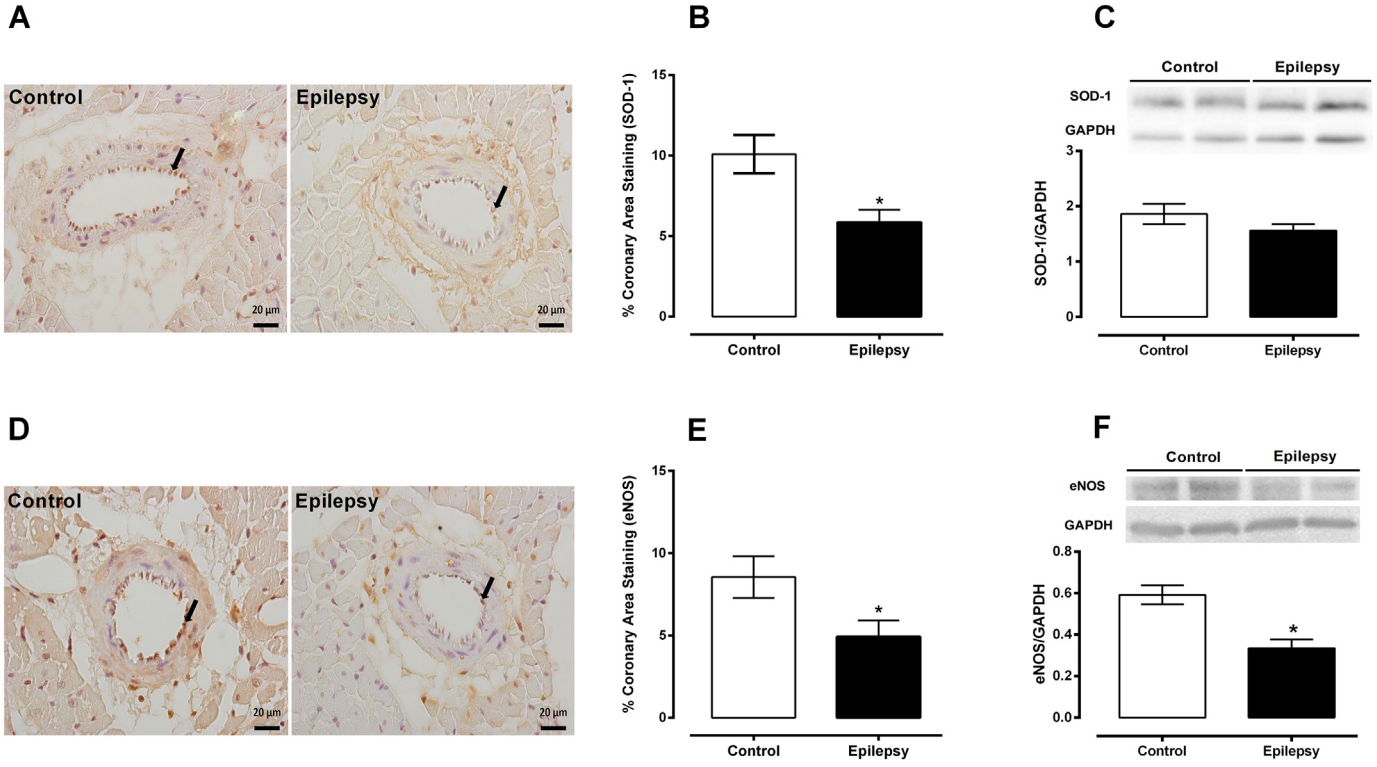

Fig. 2. Photomicrography of SOD-1 (A) and eNOS (D) immunostaining in coronary of control (left) and epilepsy (right) groups (bar = 20 µm). Mean percentage of coronary area of SOD-1 (B) and eNOS (E) staining in control and epileptic groups. Protein expression of SOD-1 (C) and eNOS (F) in the left ventricle of both control and epilepsy groups. The results are presented as mean ± SEM. *$p < 0.05$ compared to control group. Arrows indicate an example of positive immunostaining.

function, reduced oxidative stress, and prevented atherosclerosis. In addition, Nanchen et al. [17] reported that increased resting HR is associated with systemic inflammation and endothelial dysfunction.

Regarding epilepsy, several clinical and experimental studies have shown that important changes in cardiovascular parameters, such as rhythm, HR, blood pressure, electrocardiographic tracing, and circulating levels of catecholamines, are found during both ictal and interictal period [4]. The exact mechanism for these alterations needs to be clarified, but it seems to be strongly related to changes in autonomic modulation. It is important to note that variations of HR, blood pressure, and circulating catecholamines are suggested as the cause of endothelial injury [14].

Although the clinical outcomes concerning resting HR in patients with epilepsy are controversial, it has been consistently demonstrated that those who died suddenly had several cardiovascular alterations, ranging from ictal HR changes to altered cardiac tissue [3,4]. Besides, most of these alterations have been related to endothelial dysfunction [10,12,14].

Therefore, as far as we know, this is the first study showing that coronary endothelial function is altered in rats with epilepsy. Also, we previously demonstrated a higher resting HR in rats with epilepsy induced by pilocarpine [9]. Since the increased HR is related to endothelial dysfunction, we hypothesized that a high resting HR, observed in experimental models of epilepsy and in clinical studies, may contribute to impaired endothelial function observed in our results.

Clearly, further experiments are needed to confirm and understand the exact mechanisms that lead to endothelial alteration. Therefore, we believe that endothelial function should be evaluated in patients with epilepsy, especially in those with higher risk for SUDEP.

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Conflicts of interests

The authors report no conflicts of interest.

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