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Involvement of galectin-1 in the prolonged hypotensive effect induced by mandibular extension in spontaneously hypertensive rats

Abstract

Two 10-minute openings of the mouth obtained with a spring device called mandibular extension applied at 10-minute intervals (2-ME treatment) induce a hypotensive effect in Spontaneously Hypertensive Rats (SHRs), producing a vasodilation previously observed in cortical parietal pial vascular districts. Herein, transcriptional analyses were performed to investigate a possible role of Galectin-1 (Gal-1) in the 2-ME treatment-induced hypotensive effects in the pial vascular district of the parietal cortex, and in an extracerebral observational point such as the thoracic aorta. Our results show that, with respect to controls, 120 minutes after 2-ME treatment, *Gal-1* expression was significantly increased in the parietal pial vascular district and doubled in the thoracic aorta. GAL-1 levels determinations showed no differences in both the parietal pial vascular district and the thoracic aorta. Present data suggest that in our experimental model of hypertension, 2 ME treatment induced a modulation of gene expression for Gal-1 in vascular districts indicating an involvement of GAL-1 in driving the hypotensive effects.

Keywords: Mandibular extension; Galectin-1; SHR; Pial vessels; Cerebral parietal area; Hypertension.

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Introduction

A non-invasive technique consisting of a 10-minutes mouth opening, which we called Mandibular Extension (ME), induces a marked reduction of arterial blood pressure in both normotensive and Hypertensive Rats (SHR) and in humans [1-3]. This effect is particularly pronounced when ME is applied twice with a 10-minutes interval, resulting in a vasodilation that lasts up to 4 hours [4]. In the brain of both normotensive and hypertensive animals [5], this vasodilation is generalized, affecting various cortical regions, including sensory (parietal) and motor (frontal) areas. We previously showed that the ME-induced vasodilation is mediated by the release of endothelial Nitric Oxide (NO) [6], without excluding the involvement of other mechanisms.

In this work, our interest was to evaluate possible involvement of the ubiquitously expressed protein Galectin-1 (GAL-1), which is involved in many cellular functions, playing a key role in promoting vasodilation of vessels [7,8]. In this regard, GAL-1 induces the proteasomal degradation of $Ca_V1.2$ channels in smooth muscle vessels [9], that were found significantly elevated in SHRs aorta and in human mammary arteries, when compared to non-hypertensive controls.

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Therefore, we investigated whether GAL-1 is involved in ME effects in SHRs.

In these rats, systolic blood pressure spontaneously increases from 4 up to 14 weeks of age, reaching 180-200 mmHg and remaining high for the rest of their lives [10].

Materials and methods

Male Spontaneously Hypertensive Rats (SHRs) (Charles River, Calco, Italia) 4-5 months aged (b.w. 250-300 g) were used. In housing, the animals were kept in a controlled environment at a constant temperature (24±1°C) and humidity (60±5%), subjected to a cycle dark/light of 12 hours and with food and water *ad libitum*.

The rats were treated according to EU Directive 2010/63 for the protection of animals used for scientific purposes and by the Local University Ethics Committee and by the Ministry of Health (authorization no. 156/2017-PR). The animals were randomly assigned to two groups: rats subjected to two mandibular extensions (2-ME, for details see below) under sedation (T-SHRs, n=5) and rats subjected only to sedation (C-SHRs, n=5).

Intraperitoneal Sodium thiopental (Pentothal Sodium, MSD Animal health) injected at the dosage of 60 mg/Kg of b.w, diluted in physiological solution at the final concentration of 35 mg/ml, was used to induce and maintain sedation.

(Figure 1) shows an outline of the experimental protocols used: T-SHRs were sedated, and after 10 min the rats underwent to two 10-minutes ME (ME1 and ME2) with a 10-minutes interval (Figure 1A-I). 120 minutes after ME2, the animals were sacrificed with a lethal dose of anaesthetic (Penthotal Sodium 0,4 ml/100 g b.w.) for removal preparations containing parietal pial vessels and the thoracic aorta.

To assess the hypertensive status of the rats, Systolic Blood Pressure (SBP) was measured at baseline after sedation, immediately after ME2 injection, and subsequently at 30, 60, and 90 minutes by a non-invasive method (Rat Tail Cuff Method Blood Pressure Systems, IITC, Life Science Inc, Los Angeles, CA, USA). C-SHRs, however, received only anesthesia (0.2 ml/100 g bw), as T-SHRs, and blood pressure measurements were acquired at the same time and from them the same tissue samples were explanted (Figure 1A-II).

To obtain preparations containing parietal pial vessels, we first prepared a cranial window at the level of the parietal bone (2 mm posterior to the bregma and 3 mm from the midline) (Figure 1B). The craniotomy was performed with a surgical stereoscope (eyepiece at 12× magnification.) and a held high-speed micromotor drill with a round engravers bit for manual control of the size. The drilling was constantly paused to reduce heat damage, edema and the bleeding around the skull. Successively, the dura mater was gently removed together with the arachnoid to expose the pial surface. Subsequently, through a cut parallel to the exposed surface, the pial layer and the glia limitans were removed. The cut was performed to obtain sections of 10 µm thick. The slices were collected and stored at -80° C until use.

In order to assess the vasodilation effect of 2ME treatment, in a T-SHR, the pial microvascular network was observed *in vivo* by fluorescence microscopy (Figure 1D), as previously described by [4].

Preparations containing parietal pial vessels or thoracic aorta were homogenized and total RNA extracted and transcribed in c-DNA. Real-Time PCR relative quantifications were performed with a Bio-Rad 384-well CFX384 RT-PCR System, as already described previously [11,12]. Gal-1 primers were designed with Roche Probefinder software (forward: ATGGCCTGTGGTCTGGT, reverse: TCACTCAAAGGCCACACACTT, NM_019904.1) whereas the primers for the housekeeping genes were obtained from the literature [12,13].

For protein analysis, 40 µg/sample of tissue lysates were resolved by 12% acrylamide gel electrophoresis and blotting was performed on PVC-membrane by the iBlot Dry Blotting System (Life Technologies, Monza, Italy). Membranes were at first incubated with polyclonal Antibody Anti-GAL-1 (Abcam Ab108389) or Anti-GAPDH (Abcam, Ab181603) overnight at 4°C and then with the secondary G-Immunoglobulin conjugated with Horse Radish Peroxidase (IgG-HRP). Visualization of the target proteins was performed by a chemiluminescence assay (Biorad, Milan, Italy) in the Uvitec Alliance System (Eppendorf, Milan, Italy) and the Optical Density (OD) of target bands measured. The results were expressed as GAL-1 OD normalized to the reference protein GAPDH OD.

Data were expressed as means ± S.E. One-way ANOVA for repeated measures was used to evaluate changes in SBP within each group considered, and two-way ANOVA for repeated measures and the Fisher post-hoc test was performed to compare the T-SHR and C-SHR groups, considering the interaction "group by time" (Figure 1C).

Unpaired t test was performed for analysis of data obtained from gene expression and protein levels.

The analysis was conducted using GraphPad Prism 7.0 software (Software Inc. San Diego CA). Significance was set at $P<0.05$.

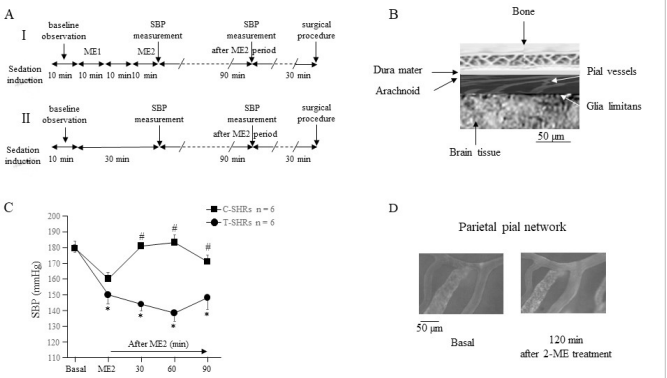

Figure 1: Schematic representation of experimental procedures. **(A)** Timing of systolic blood pressure measurements in the 2-ME experiments (AI) and in controls (AII). After ME2, blood pressure recordings were done every 30 minutes. **(C)** Values observed in T-SHRs and C-SHRs at the end of the baseline observation period, at the end of ME2 application and at 30, 60, 90 minutes after ME2. **(B)** Representation of a portion of the parietal area in an adult SHR, where we visualized the pial microcirculation. **(D)** Computer-assisted images of pial microvascular network on the left parietal cortex of a SHR, acquired before 2-ME treatment (Basal) and 120 minutes after ME2.

*significant difference *vs.* baseline; #significant difference between groups at the same time point.

Results

In T-SHRs, 2-ME treatment caused a significant reduction in SBP respect to baseline (180.0±3.3 mmHg) starting from the end of ME2 (150.0±6.0 mmHg; $F_{5,20}$=1.329, P<0.037) up to 90 minutes post-ME2 (149±7 mmHg; P=0.016), reaching a nadir at 60 minutes post ME2 (139.0±6.0 mmHg; P=0.009). In C-SHRs, no significant changes were recorded ($F_{3,12}$=1.791, P=0.202). Moreover, a significant difference between the two groups was observed ($F_{1,5}$=68.41, P=0.0004), as was a significant group/time interaction at 30, 60, 90 minutes post-ME2 ($F_{4,20}$=12.76, P<0.0001; P<0.0001, P<0.0001 and P=0.0014 respectively) (Figure 1C).

In a T-SHR subjected to 2-ME treatment we observed a vasodilation at the pial microcirculation level in the parietal cortex that accompanied the blood pressure reduction (Figure 1D), confirming previous observations [4].

Given the vasodilation associated with the hypotensive effect of 2-ME treatment, we carried out a transcriptional analysis in order to evaluate the expression of *Gal-1* at the brain level, in preparations containing parietal pial vessels. 120 minutes after ME2, the *Gal-1* expression was significantly higher in T-SHRs (1.575±0.146) respect to C-SHRs (0.878±0.081; P=0.003, t=4.432, DF=7) (Figure 2A), We tested the *Gal-1* expression also in an out-of-brain observational point, such as the thoracic aorta (Figure 2A). In this case, the expression of *Gal-1* was not significantly different in T-SHRs (2.253±0.948) and C-SHRs (0.918±0.174; P=0.1172, t=-1.828, DF=6). However, the expression ratio T-SHRs / C-SHRs was 2.5 suggesting a possible modulation of ME on the expression of *Gal-1*.

The analysis of the protein levels of GAL-1 in both preparations containing parietal pial vessels (T-SHRs: 1.128±0.144; C-SHRs: 1.430±0.214) and thoracic aorta (T-SHRs: 1.155±0. 409; C-SHRs: 1.217±0.356) showed no significant differences between the two groups (P=0.268, t=1.218, DF=6 for preparations containing parietal pial microcirculation; P=0.564, t=-0.627, DF=4 for thoracic aorta) (Figure 2B).

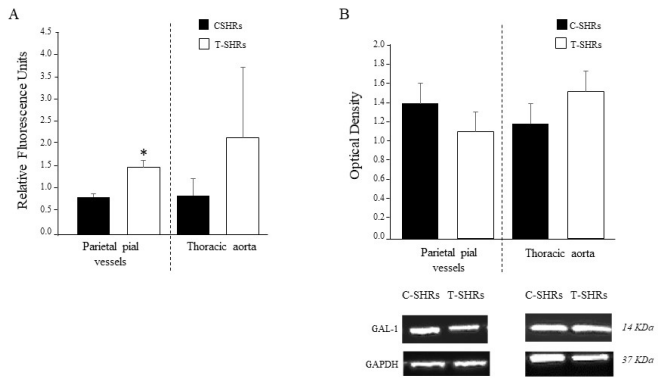

Figure 2: Expression of *Gal-1* and Protein levels. **(A)** In parietal pial vessels *Gal-1* expression significantly increased after 2-ME treatment, while in thoracic aorta doubled, thought did not reach statistical significance. **(B)** Protein levels of GAL-1 in parietal pial vessels and in thoracic aorta. Histograms represent the Optical Density (OD) of target GAL-1 normalized to the OD of the reference GAPDH. Lower, examples of GAL-1 and GAPDH blots are also reported. Data are expressed as mean ± SEM. *P=0.003.

Discussion/conclusion

This study confirms the hypotensive effect induced by 2-ME in anesthetized Spontaneously Hypertensive Rats (SHRs) and extends previous findings by investigating the underlying vasodilatory mechanisms.

We tested the possible role played by Gal-1, a protein known for its various neuroprotective [14,15] and vasodilator effects [9] able to reduce calcium entry into vessel smooth muscle cells. In blood vessels, Gal-1 binds $Ca_V1.2$ channels, promoting their ubiquitination, leading to a reduction in calcium uptake and oscillation of its gradient, thus decreasing the contraction of smooth muscle cells and inducing vasodilation [9].

Of interest are the effects of Gal-1 in promoting angiogenesis [16-18] and as a protective factor in normal cardiac homeostasis and post-infarction remodeling by preventing cardiac inflammation [19]. Thus, Gal-1 treatment represents a potential novel strategy to attenuate heart failure in acute myocardial infarction [19].

A recent study [20] was conducted on human atherosclerotic plaques comparing them with samples of healthy aortic wall. Gal-1 staining was prominent in the tunica media of control aortas, which is mainly composed of α-Smooth Muscle Actin (α-SMA)-positive, whereas it was significantly decreased in human atherosclerotic plaques, suggesting that Gal-1-driven circuits may be potential therapeutic strategies in atherosclerosis.

In this work we evaluated the gene and protein expression of Gal-1, in order to assess whether 2-ME treatment could influence the regulation of GAL-1 levels both in the pial vascular district of the parietal cortex and in the thoracic aorta, as an extracerebral observational point in SHRs.

After 2-ME treatment, in the pial parietal vascular district, *Gal-1* gene expression resulted significantly increased in comparison with the controls. In the thoracic aorta, *Gal-1* expression was doubled in 2-ME-treated rats compared to controls, but the difference did not reach statistical significance.

In both the parietal pial vascular district and thoracic aorta, GAL-1 levels did not significantly change after 2-ME treatment. The different trend observed between *Gal-1* expression and GAL-1 protein levels could be due to the fact that both were analysed in the same samples, obtained 120 minutes after the end of the 2-ME treatment, while a modulation of protein expression could take longer to be quantifiable.

Overall, our results suggest that 2ME affects Gal-1 expression, which likely drives the hypotensive response in this model of hypertension.

Therefore, in future studies it will be interesting to evaluate the gene and protein expression of Gal-1 in other peripheral districts well supplied with arterioles, such as the skin or the abdominal wall to obtain additional information on the role played by Gal-1 in the regulation of blood pressure.

Declarations

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Conflict of interest: The authors declare that they have no known competing financial interests or personal relationships that could appear to influence the work reported in this paper.

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