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Pretreatment with relaxin does not restore NO-mediated modulation of calcium signal in isolated coronary endothelial cells isolated from spontaneously hypertensive rats

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Abstract

We demonstrated that in coronary endothelial cells (RCEs) from normotensive Wistar Kyoto rats (WKY), the hormone relaxin (RLX) can increase NO production and reduce calcium transient by a NO-related mechanism. Since an impairment of the NO pathway has been demonstrated in the spontaneously hypertensive rat (SHR), in the present study we aimed at exploring the effects of RLX on RCEs isolated from SHR, hypothesizing that RLX could restore calcium responsiveness to NO.

Experiments were approved by the Animal Subjects Review Board of the University of Florence. RCEs were isolated from WKY and SHR. Calcium transient was evaluated by image analysis after the administration of angiotensin II (AT II).

AT-II (1 μM) caused a prompt rise of $[Ca^{2+}]i$ in WKY and SHR RCEs and a rapid decrease with a decay time higher in SHR than in WKY. NOS inhibition increased calcium transient in WKY, but not in SHR RCEs. Whereas RLX pretreatment (24 h, 60 ng/ml) was ineffective in SHR, it strongly reduced calcium transient in WKY in a NOS-dependent way.

The current study offers evidences that RLX cannot restore NO responsiveness in SHR RCEs and suggests an accurate selection of patients eligible for RLX treatment of cardiovascular diseases.

Keywords: Angiotensin II, cGMP-dependent protein kinase I, cardiovascular diseases, NG-nitro-L-arginine methylester, normotensive Wistar Kyoto rats, S-nitroso-N-acetylpennicillamine, W1400

Introduction

In the vascular system, the NITRIC OXIDE (NO) pathway mediates vasorelaxation and platelet anti-aggregation and protects from ischemic disorders [1]. NO, physiologically produced by different nitric oxide synthase isoforms (eNOS, nNOS and iNOS), can activate soluble guanylyl cyclase (sGC) to produce cyclic guanosine monophosphate (cGMP) which in turnactivates the cGMP-dependent protein kinase (cGK-I), and modulates ion channels, and phosphodiesterases [2]. In this context, convincing evidence exists in the literature that the hormone relaxin (RLX) can promote coronary and systemic vasodilatation by increasing NO bioavailability and NOS enzyme expression [3], thereby reducing hypertension and protecting the heart against ischemia/reperfusion-induced injury [4,5]. Thus, administration of recombinant human H2 RLX, or serelaxin, has been proposed as a potential therapeutic strategy for hypertension and heart ischemia [6].

The spontaneously hypertensive rat is an animal model widely used for the study of hypertension, hypertensive heart disease and cardiac hypertrophy. In this model, alterations in Ca^{2+} handling have been described at very early stages of the disease, even before the appearance of HF [7]. Many different factors have been found to be involved in this spontaneous, age-dependent pathological condition, including an impairment of the NO pathway. In particular: i) mRNA expression of cGMP-dependent protein kinase I (cGKI) was found reduced in aortic rings of 6 week-old SHR [8]; ii) decreased cGK activity was detected in ventricular and atrial tissue of aged SHR [9] and other forms of hypertensive animals [10] and iii) cGKI expression is reduced in cardiomyocytes and coronary endothelial cells (RCEs) of 12 week-old SHR and according to our research data, the cGKI expression is reduced in cardiomyocytes and coronary endothelial cells (RCEs) of 12 week old SHR [11, 12]. Of note, cGKI is a major regulator of intracellular calcium homeostasis and its induced over-expression was found to restore NO-mediated calcium regulation in aortic smooth muscle cells and coronary endothelial cells (RCEs) isolated from SHR [13] and RCEs [12].

Along this line of thought, administration of RLX to SHR was reported to cause a sustained decrease in blood pressure and to substantially blunt the vascular response to vasoconstrictors [14, 15]. Besides these acute functional effects, RLX was also capable of reverting arterial adverse remodeling and decreased compliance in elderly SHR [16].

The cellular mechanisms underlying these vascular effects of RLX are not fully understood but represent matter of investigation because of their obvious medical interest. In this context, previous studies performed by our team on RCEs from normotensive Wistar Kyoto (WKY) rats, the normal counterpart of SHR, have demonstrated that RLX could increase NO production by up-regulating NOS expression and decrease vasoconstrictor-induced $[Ca^{2+}]i$ rise by a NO-related mechanism [17]. Therefore, it was reasonable to assume that a similar mechanisms may also be operating in SHR.

In the present study we aimed at exploring the effects of RLX on RCEs isolated from SHR, based on the working hypothesis that RLX may restore [Ca^{2+}]i responsiveness to NO. According to our previous study on normotensive rats, we used angiotensin II (AT-II) to induce [Ca^{2+}]i increase in RCEs, since these cells have been shown to express AT-II receptors and to respond to exogenous AT-II by modulation of NO-dependent [Ca^{2+}]i increase [12,18]. Preliminary data were presented at the Fourth International Conference on "Relaxin and related peptide" [19].

Results

Intracellular Ca^{2}: control conditions - At baseline, [Ca^{2+}]i, evaluated by Fura 2 fluorescence, was 112.5±2.76 nM in RCEs isolated from WKY and slightly higher in those from SHR (144.1±7.43 nM). Stimulation of RCEs with 1 µM AT-II, caused a prompt rise of [Ca^{2+}]i in both WKY and SHR strains (Fig. 1).

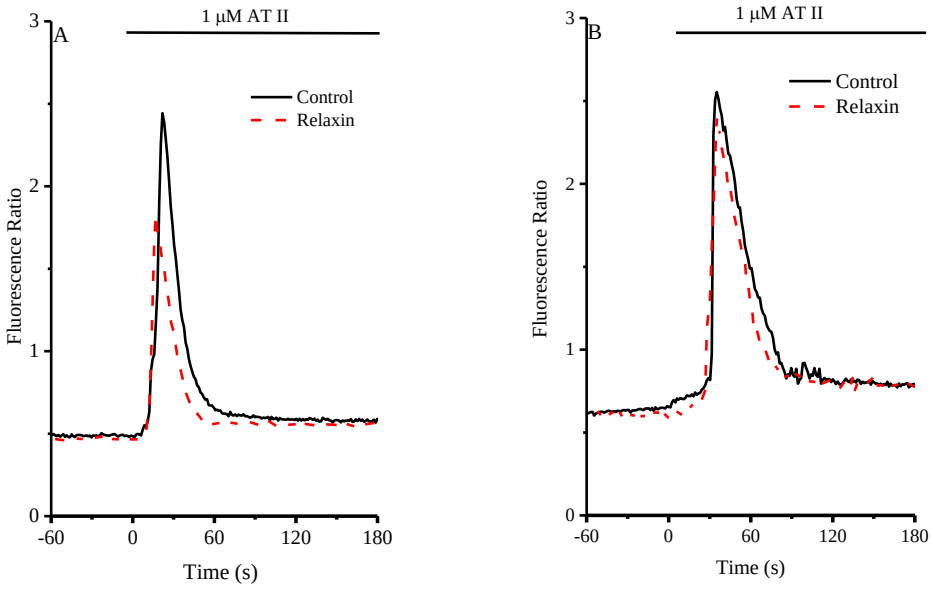

Figure 1. Evaluation of [Ca2+]i. in RCE isolated from WKY and SHR rats A) Representative tracings of [Ca2+]i-associated fluorescence in Fura-2 RCEs from WKY rats in control and RLX-treated cultures upon challenge with AT-II. **B)** Representative tracings of [Ca2+]i-associated fluorescence in Fura 2-loaded RCEs from SHR in control and RLX-treated cultures upon challenge with AT-II. AT-II (1 µM) was added at the arrow (time = 0) and maintained throughout the experiment, as indicated. Cells were incubated for 24 h in serum-free medium in the absence (controls) or presence of 60 ng/ml RLX.

In the SHR-derived cells, the maximum [Ca^{2+}]i increase was slightly, albeit not significantly higher than in those from WKY (Fig. 2 A). Calcium signal decreased rapidly in WKY-derived RCEs with a decay time of 19.2±0.61 s, whereas in SHR-derived cells, the decay time was significantly higher (Fig. 2B). A 10-min. incubation with the NO-donor SNAP significantly decreased maximum [Ca^{2+}]i increase and decay time in the WKY-derived cells, whereas it was ineffective in the SHR-derived ones (Fig. 2). Moreover, 20-min. preincubation with the non-selective NOS inhibitor L-NAME significantly increased maximum [Ca^{2+}]i increase and decay time in the WKY-derived, but not in the SHR-derived cells. Inhibition of NOS II with W1400 had no effect on calcium transient in both strains.

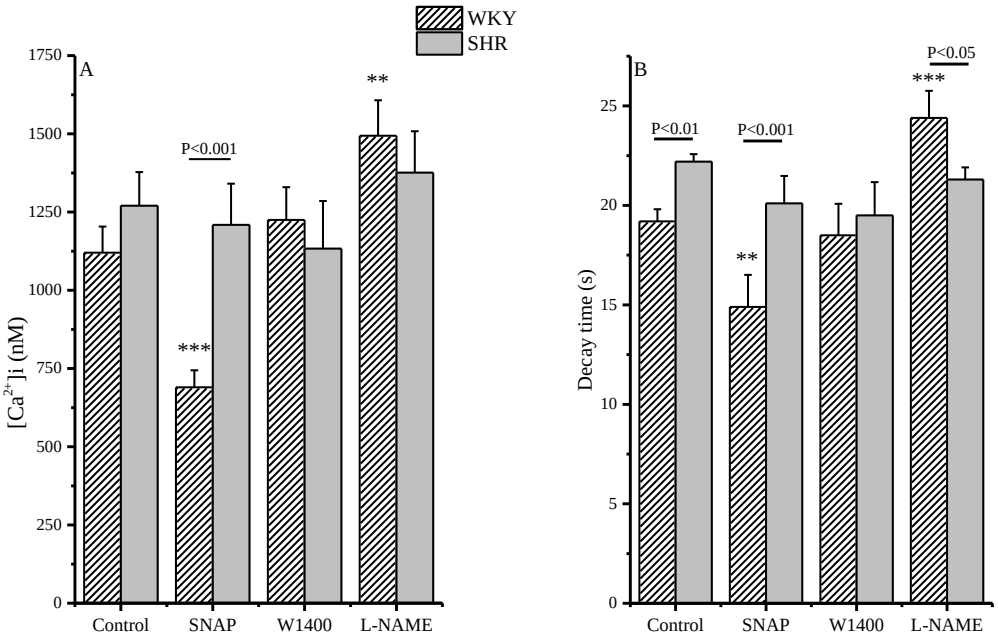

Figure 2. Evaluation of [Ca^{2+}]i in RCE isolated from WKY and SHR rats. [Ca^{2+}]i increase (peak, A) and [Ca^{2+}]i transient decay time (B) in WKY- and SHR-derived RCEs. SNAP (100 μM), L-NAME (10 μM) or W1400 (1 μM) were incubated 20 min before the administration of AT-II (1 μM). Values are the mean (± S.E.M.) of data from three separate RCE cell isolations, where at least 20-24 cells for each type of protocols were analyzed separately. Segments on the top of bars indicate significance between WKY and SHR; stars indicate significance between treatment and control, same strain: ***$P < 0.001$; **$P < 0.01$

Intracellular [Ca^{2+}]: RLX effects - In WKY-derived RCEs, a 24-h incubation with RLX decreased both peak and decay time of the agonist-induced [Ca^{2+}]i transient (Fig. 1A, Fig. 3): the effect of RLX preincubation was potentiated by a 10-min. incubation with SNAP. A 20-min. incubation with either the nonspecific NOS inhibitor L-NAME or the selective NOS II inhibitor 1400W modified the kinetic of AT-II induced [Ca^{2+}]i transient in RLX-treated WKY-derived RCEs: as shown, both inhibitors were able to increase the maximum and the decay time of the calcium transient. A similar 24h incubation with RLX in SHR-derived cells was ineffective in reducing the calcium transient, as evaluated by maximum and decay time. Again, in SHR-derived cells, the short incubation with SNAP or with both NOS inhibitors did not modify calcium transient in the RLX-pretreated cells.

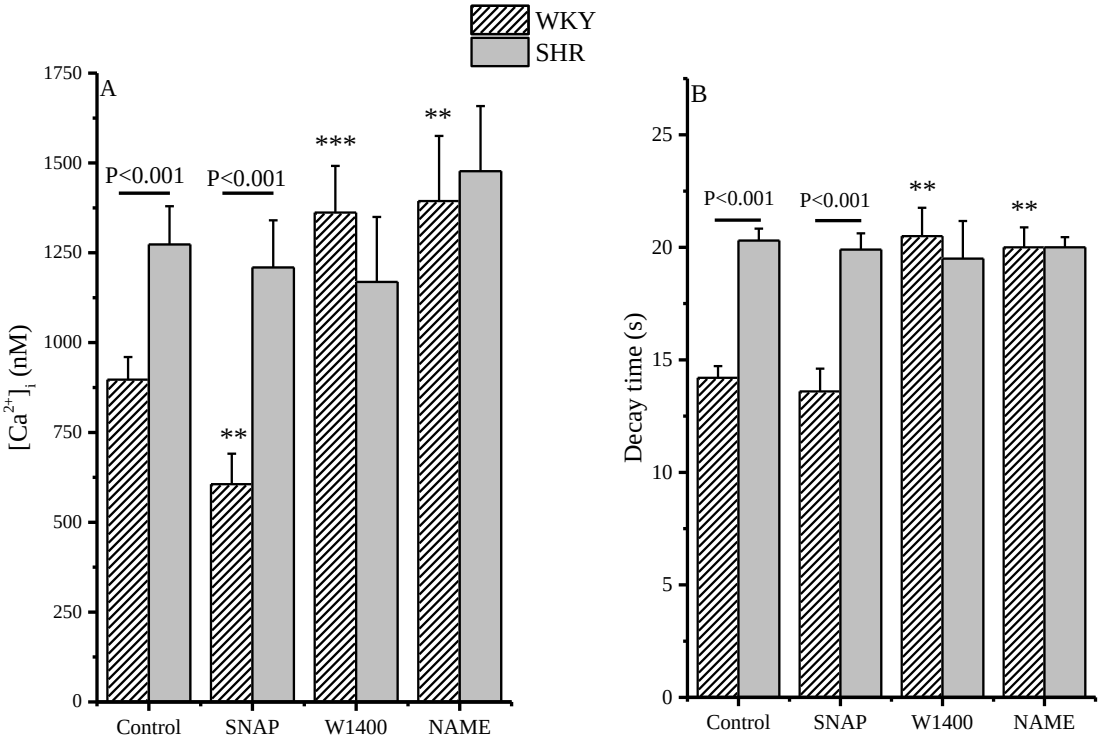

Figure 3. Evaluation of [Ca^{2+}]i in RCE isolated from WKY and SHR rats pretreated with RLX. [Ca^{2+}]i increase (peak, A) and [Ca^{2+}]i transient decay time (B) in WKY- and SHR-derived RCEs. Cells were pretreated with RLX (60 ng/ml) for 24 h; SNAP (100 µM), L-NAME (10 µM) or W1400 (1 µM) were incubated 20 min. before the administration of AT-II (1 µM). Values are the mean (± S.E.M.) of data from three separate RCE cell isolations, where at least 20-24 cells for each type of protocols were analyzed separately. Segments on the top of bars indicate significance between WKY and SHR; stars indicate significance between treatment and control, same strain: ***P < 0.001; **P < 0.01

Discussion

The present data confirm that $[Ca^{2+}]i$ in RCEs isolated from normotensive WKY rats is strongly modulated by the NO pathway. In particular, while a NO donor decreases the calcium transient induced by AT-II, an aspecific inhibitor of NOS (L-NAME) can increase it. Moreover, the hormone RLX, known for its vasodilatory properties [5], also reduces calcium transient: this effect appear to be related to NOS II induction and enhanced endogenous NO generation [17], as can be judged by the effectiveness of the NOS II inhibitor W1400, which is ineffective in the control cells not treated with RLX. These data confirm the important role of NO in endothelial cells as modulator of calcium signal in physiologically normal rats and suggest that NO can act in an autocrine manner [17]. A remarkably different behavior is observed in SHR-derived RCEs. In fact, in these cells, NO is unable to modulate $[Ca^{2+}]i$ and this ineffectiveness is maintained after treatment with RLX, suggesting that a downstream step of the NO pathway is altered. In this context, we have previously reported that SHR-derived RCEs show low/absent expression of cGKI enzyme [12]. Moreover, a similar reduction of cGKI has been described in cardiomyocytes [11] and aortic smooth muscle cells [13]. The present data that RLX is unable to restore NO responsiveness in SHR-derived RCEs appears to be at variance with previous *in vivo* studies in which RLX reduced blood pressure when administered to SHR [14]. This discrepancy may depend on many reasons, including that, *in vivo*, RLX could directly exert its effects on vascular smooth muscle cells, whose contractility is inhibited by RLX [24]. Of note, a direct NO-mediated relaxant effect of RLX on smooth musculature has been consistently described in other target organs, including the uterus [25,26] and the gastrointestinal tract [27].

The current study, while offering additional evidences that RLX cannot restore NO responsiveness in SHR-derived RCEs, further underlines the importance of the NO/sGK/cGKI pathway in controlling the $[Ca^{2}]i$ dynamics presiding to the regulation of vascular tone. This notion may have clinical implications, as it suggests that caution is required in identifying the cohort of patients that may be successfully treated with NO-modulating drugs for cardiovascular diseases. A genome-wide association study found that the common genetic variants in human cGKI-1 gene (*PRKG1*) are significantly associated with changes in diastolic blood pressure in response to an acute salt load in patients with hypertension [28]. Chronically elevated blood pressure increases left ventricular (LV) load, enhances LV radial systolic performance and leads to LV hypertrophy. Recently, LV systolic radial function has been associated with common polymorphisms in *PRKG1* [29]. In particular, LV radial systolic deformation (strain) is significantly higher in GAT homozygotes of *PRKG1*gene than in heterozygotes and noncarriers. Even if further studies must be undertaken to elucidate how the

genetic variants of *PRKG1* might influence cardiovascular diseases, these data suggest that NO-enhancing drugs and RLX in these patients could negatively influence the correct clinical evaluation of their actual efficacy in the treatment of hypertension and hypertensive heart diseases.

Materials and Methods

Chemicals

Highly purified porcine RLX (2,500-3,000 U/mg) was the generous gift of O. D. Sherwood. RLX was used at a concentration of 60 ng/ml, which is in the range of that found effective in inducing coronary vasodilatation in rat hearts [20]. Media, sera, and reagents for cell culture were from Sigma-Aldrich (Milan, Italy) and Gibco Life Technologies (Milan, Italy). Cell culture plastic ware was purchased from Costar (Corning Costar Co., Costar Italia, Milan, Italy). Fura 2-AM and Pluronic F127 were from Molecular Probes Life Technologies (Milan, Italy). The selective NOS II inhibitor 1400W [21] was from Alexis Biochemicals (Enzo Life Sciences, New York, U.S.A.) and the NO-donor S-nitroso-N-acetylpennicillamine (SNAP) from Tocris (Bristol, U.K.). NG-nitro-L-arginine methylester (L-NAME) and angiotensin II (AT II) were from from Sigma-Aldrich (Milan, Italy) as were the other chemicals used.

Isolation and culture of rat coronary endothelial (RCE) cells

RCEs were isolated from the heart of 3-4-month old male Wistar Kyoto and aged matched SHR rats, as described previously [22]. Rats (Charles River, Lecco, Italy) were housed in the Centro per la Stabulazione degli Animali da Laboratorio (Ce.S.A.L., University of Florence), maintained for at least one week after their arrival in a 12 hours dark-light cycle with pellet food and water *ad libitum*. Formal approval to conduct the experiments described was obtained from the Animal Subjects Review Board of the University of Florence. The ethics policy of the University of Florence complies with the Guide for the Care and Use of Laboratory Animals of the U.S. National Institutes of Health (NIH Publication No. 85-23, revised 1996; University of Florence Assurance No. A5278-01).

Briefly, after enzymatic digestion of the heart, the suspension was centrifuged and the pellet was stirred for 30 min at 37°C in the presence of 10 mg/50 ml trypsin. The recovered pellet was resuspended in 15 ml of culture medium (see below), and plated. After 4 h, cells were washed twice and grown until confluence (5-6 days) in M199 containing 10% fetal calf serum (FCS), 10% newborn calf serum, 250 U/ml penicillin G, 0.625 µg/ml amphotericin, and 250 µg/ml streptomycin. Isolated RCEs were cytocharacterized as previously reported [22,23]. Cells

immunoreactive for endothelial markers ranged between 96% and 98%. For all experiments, cells were used at the first passage. Stimulation of RCEs with RLX was carried out in M199 medium without phenol red.

Determination of intracellular Ca^{2+} Intracellular cytosolic Ca^{2+} ($[Ca^{2+}]i$) was evaluated with Fura-2 by microscopic image analysis as described previously [12,22]. Briefly, cells were grown on round cover slips to subconfluence and then incubated for 24 h in serum-free medium in the absence (controls) or presence of RLX (60 ng/ml). Cells were loaded with the Ca^{2+}-sensitive fluorescent probe Fura 2-AM (4 µmol/l) and Pluronic F (0.02%) for 45 min at room temperature in HEPES-bicarbonate buffer containing (mM): NaCl 140, KCl 2.9, NaH_2PO_4 0.5, $NaHCO_3$ 12, $MgCl_2$ 0.9, HEPES 10, $CaCl_2$ 1, glucose 10, adjusting pH to 7.4 with NaOH 1 N.

Coverslips were washed and mounted in the stage of an epifluorescence inverted microscopy in a perfusion chamber warmed at 35°C. $[Ca^{2+}]i$ was measured dynamically by monitoring Fura-2 fluorescence ratio. Consecutive images, obtained with excitation wavelengths of 340 and 380 nm and an emission wavelength of 510 nm, were recorded with a charge coupled device (CCD) video camera and digitized by an analogical/digital converter (resolution: 256×256 pixels, time interval between subsequent images: 800 ms). Image ratios (340/380 nm) were obtained every 3 s on a pixel-to-pixel basis after subtraction of the background.

After measurements of $[Ca^{2+}]i$ in basal conditions, 1µM AT II was added directly into the perfusion chamber, and the time course of $[Ca^{2+}]i$ increase induced by the agonist was analyzed for at least 15 min as already described [12,22]. A noise/signal ratio of 0.5 of 340/380 nm fluorescence was considered as the lowest detectable limit for $[Ca^{2+}]i$ transient. In some experiments, 100 µM SNAP, the broad-spectrum NOS inhibitor L-NAME (10 µM) or the specific NOS II inhibitor 1400W (1 µM) were added to the perfusion chamber 20 min before the addition of the agonist. Calibration curves were performed using a Kd of 214 nM for Fura-2.

The experimental data were exported as ASCII file format and graphically elaborated with MicroCal Origin 9 software (OriginLab, Northhampton, MA). The decay time of calcium transient was calculated as reported previously [22] and expressed as the time (s) needed for fluorescence ratio to reach 36.79% of the maximal value. The reported values are the mean (± SE) of data from three separate RCE cell isolations, where at least 20-24 cells for each type of protocols were analyzed separately.

Calculations and statistical analysis

Statistical comparisons were performed by using one-way ANOVA test followed by Student-Newman-Keuls multiple comparison test. A *P value* ≤ 0.05 was considered as the low level of significance.

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Author Contributions

P.F. and D.B. conceived and designed the experiments; S.N., L.D.C.M and M.Z. performed the experiments; P.F. and L.D.C.M. analyzed the data; C.G. contributed reagents and analysis tools; P.F. and D.B wrote the paper.

Conflicts of Interest

The authors declare no conflict of interest.

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