Original Paper

The Role of β -Adrenergic Overstimulation in the Early Stages of Renal Injury

Mariana Charleaux de Ponte^a Fernando Augusto Malavazzi Casare^a
Juliana Martins Costa-Pessoa^a Vanessa Gerolde Cardoso^a Gerhard Malnic^a
Margarida Mello-Aires^a Rildo Aparecido Volpini^b Karina Thieme^c
Maria Oliveira-Souza^a

^aLaboratory of Renal Physiology, Department of Physiology and Biophysics, Institute of Biomedical Sciences, ^bLaboratory of Basic Kidney Disease (LIM-12), Nephrology Department, Medical School,

^cLaboratory of Carbohydrates and Radioimmunoassays (LIM-18), Medical School, University of São Paulo, São Paulo, Brazil

Key Words

Isoproterenol • β -adrenergic stimulation • Renal hemodynamics • Oxidative stress • Inflammation • Intrarenal renin-angiotensin system

Abstract

Background/Aims: To assess the possible contribution of the β -adrenergic overstimulation in early stages of renal injury, the present study evaluated, in rats, the effects of the β -adrenoceptor agonist isoproterenol (ISO) on renal function and morphology, as well as the renal mRNA and protein expression of the NADPH oxidase isoform 4 (Nox 4) and subunit p22^{phox}, endoplasmic reticulum (ER) stress, pro-inflammatory, pro-apoptotic and renin-angiotensin system (RAS) components. **Methods:** Wistar rats received ISO (0.3 mg.kg⁻¹.day⁻¹ s.c.) or vehicle (control) for eight days. At the end of the treatment, food and water intake, urine output and body weight gain were evaluated and renal function studies were performed. Renal tissue was used for the morphological, quantitative PCR and immunohistochemical studies. **Results:** ISO did not change metabolic parameters or urine output. However it induced a decrease in renal blood flow and an increase in the filtration fraction. These changes were accompanied by increased cortical mRNA and protein expression for the renal oxidative stress components including Nox 4 and p22^{phox}; ER stress, pro-inflammatory, pro-apoptotic as well as RAS components. ISO also induced a significant increase in medullar renin protein expression. **Conclusion:** These findings support relevant information regarding the contribution of specific β -adrenergic hyperactivity in early stage of renal injury, indicating the reactive oxygen species, ER stress and intrarenal RAS as important factors in this process.

© 2017 The Author(s)
Published by S. Karger AG, Basel

Introduction

In physiological condition, the sympathetic nervous system (SNS) provides a critical aspect in the maintenance of homeostasis through the cardiac function and peripheral resistance control. In this condition, the blood pressure and the vascular tone ensure specific tissues perfusion [1]. The SNS action depends on the release of neurotransmitters, such as catecholamines, that interact with specific receptors named α -adrenergic (α -AR) and β -adrenergic (β -AR) located in target tissues [2]. The kidneys are innervated by efferent sympathetic nerve fibers, which preferentially release norepinephrine (NE) and contact the wall of the afferent arteriole and tubules [3], an essential condition that regulates the renal hemodynamic and fluid balance. The β -AR types 1 and 2 have been detected in vascular smooth muscle cells from arteries and arterioles, as well as in proximal and distal tubular cells [4]. Furthermore, the β 1-AR is expressed in the juxtaglomerular granular cells, where it regulates the NE action on the renin secretion [5].

Sympathetic hyperactivity is commonly associated with progression of both cardiovascular and chronic kidney diseases [6, 7]. Although there is no evidence on the specific involvement of β -AR stimulation in the progression of chronic kidney disease, *in vitro* studies demonstrated that in human embryonic kidney (HEK) cells, the β 2-AR signaling is closely related to activation of NADPH oxidase and generation of intracellular reactive oxygen species (ROS) [8]. In addition, NE or isoproterenol acting via β -ARs, stimulates the angiotensinogen gene expression in opossum kidney (OK) proximal tubular cells [5, 9]. These findings are relevant and indicate that in animal models both NE and isoproterenol via β -ARs can stimulate the intrarenal RAS, as well as ROS generation. Furthermore, in various tissues increased ROS production is closely related to the endoplasmic reticulum (ER) stress, inflammatory, fibrotic and apoptotic responses [10-12], predictive factors for the progression of chronic kidney disease [10, 13].

In line with these findings, isoproterenol (ISO) or isoprenaline is a synthetic catecholamine and non-selective β -AR agonist of clinical use, that acutely increase the heart rate and enhance sinoatrial and atrioventricular conduction [14]. However, in animal models, the chronic treatment with ISO causes severe stress to the cardiomyocytes resulting in hypertrophy and myocardial infarct, which are similar to those observed in human [15, 16] and are closely related to oxidative stress and inflammation [17, 18]. In contrast, there are no evidences of the effects of ISO treatment on renal pathophysiology. This is an important question with clinical relevance and limited advance in our knowledge base. Thus, the main purpose of this study was to evaluate if eight-day treatment with ISO induces changes on renal function and morphology in rats, as well as intrarenal oxidative stress, ER stress, inflammation, apoptosis and RAS components expression. Together, these factors can lead to the early stages of renal injury induced by specific β -ARs overstimulation.

Materials and Methods

Animals and study design

All procedures and protocols used in this study were approved by the Institutional Animal Care and Use Committee of the University of São Paulo (protocol n° 71, page 20/03). Male Wistar rats ($n = 22$), weighing 250 - 300 g) were obtained from the animal care facility of the Department of Physiology and Biophysics, Institute of Biomedical Sciences, University of São Paulo (São Paulo, Brazil). All animals were housed at the department facility under standard conditions (constant temperature of 22°C, 12:12-h light-dark cycle, and 60% relative humidity). The rats were fed standard rat chow and provided water *ad libitum*. The animals were randomly allocated into the following two groups: control rats ($n = 10$), which received only vehicle (0.1 ml of soy bean oil, s.c.) and Isoproterenol-treated rats ($n = 12$), which received (-)-ISO (0.3 mg.kg⁻¹.day⁻¹, resuspended in 0.1 ml of soy bean oil, s.c.) (Sigma Aldrich, St. Louis, USA). The rats were treated every 24 hours for eight days.

Physiological parameters

On the eighth day of treatment, the animals were housed individually in metabolic cages for 24 hours and the food (g/day) and water (ml/day) ingestion, as well as urine output (ml/day) were monitored. The body weight (BW) gain was calculated as following: (final BW - initial BW).

Renal function studies

At the end of the treatment, the animals were anesthetized with zoletil (50 mg/kg zolazepan and 50mg/kg tiletamine) and xylazine (5 mg/kg) placed on a warm table to maintain body temperature and tracheostomized using a PE-260 tube to maintain ventilation. As previously described [19, 20] and summarized here, the right carotid artery and right jugular vein were cannulated using a PE-50 catheter (Clay Adams, Franklin Lakes, NJ) for blood sampling and continuous fluid infusion, respectively. The urinary bladder was catheterized using a PE-260 via a suprapubic incision for urine collections. Before clearance experiments, the first urine sample (~1 ml) was collected directly from the urinary bladder of each animal for further analysis of osmolality and determination of electrolyte content. A blood sample was also collected for osmolality and electrolyte analyses. Then, renal hemodynamic and function measurements were performed as described below. The animals received infusion of a isotonic solution (0.9 M NaCl) for 30 min (0.1 ml/min), using an infusion pump (Harvard Instruments, Holliston, MA), followed by infusion of a 1ml bolus containing 90 mg/animal of inulin (Sigma-Aldrich, St. Louis, MO) and 2 mg/animal of sodium para-amino-hypuric acid (PAH; Sigma-Aldrich). After that, 30 min of continuous infusion with control solution containing 15 mg/ml inulin, and 4 mg/ml PAH at 0.1 ml/min (Sigma-Aldrich) was performed to stabilize the experiment. Then, under the same continuous infusion, four consecutive 30-minute urine and arterial blood sample (500 μ l) collections were obtained to determine the kidney function.

Plasma and urine inulin and PAH concentrations were evaluated by standard colorimetric techniques using a spectrophotometer (μ Quant; Bio-Tek Instruments Inc, Winooski, USA). The renal plasma flow (RPF) and glomerular filtration rate (GFR) were calculated from the PAH [21] and inulin [22] clearance, respectively. The filtration fraction (FF) was obtained as the GFR/RPF ratio and expressed as a percentage (%). The urine volume of each sample was determined for the urine flow measurement (V). The clearance was calculated using the following formula, where C is clearance, x is the urine or plasma concentration of a substance, and V represents urine flow rate: $C = (\text{Urine}_x \cdot V) / \text{plasma}_x$.

Plasma and urine analysis

The plasma and urine osmolality were measured using an osmometer (Precision Systems, Natick, MA), and sodium concentrations were determined by flame photometry (9180 Electrolyte Analyzer; Roche, Basel, Switzerland). The fractional sodium excretion (FE%) was calculated using the formula: $(V [\text{Na}^+]_u / [\text{Na}^+]_p \cdot \text{GFR}) \times 100$; where V is urine flow rate, $[\text{Na}^+]_u$ is the urine sodium concentration and $[\text{Na}^+]_p$ is the plasma sodium concentration. The urine protein and creatinine concentrations were also evaluated using the metabolic cage sample and colorimetric tests (Labtest, Lagoa Santa, Brazil). The experiments were carried out following the manufacturer's instructions and urine protein concentration was normalized by 24-hour urine creatinine.

Morphological studies

After the renal function experiments, the kidneys were immediately perfused with PBS (0.15 M NaCl containing 10 mM sodium phosphate buffer, pH 7.4) at 20 ml/min through the distal aorta using a peristaltic perfusion pump (Milan Scientific Equipment, BP 600 PR, Brazil), as previously described [20]. At this condition, euthanasia was performed by exsanguination. One kidney per rat was isolated, removed, weighed and used for the quantitative PCR studies. The remaining kidney was fixed in 4% paraformaldehyde solution, dehydrated and embedded in paraffin for morphological studies. For these assays, 4- μ m-thick kidney sections were stained using *Verhoeff* method and renal morphology was evaluated blindly by two independent persons by using a light microscope (Eclipse 80i, Nikon). For the planar glomerular area analysis, all glomeruli with apparent macula densa and afferent arterioles from the renal cortical area of each rat were included. The area of each glomerulus was determined, and the mean glomeruli areas were obtained using a morphometry software (NIS-Elements D, Nikon, Tokyo, Japan).

Ventricles and kidney weights

Both heart ventricles and one kidney were excised and weighed. The ratio between ventricles or kidney weight (mg) and body weight (g) was used as an index to confirm cardiac or renal hypertrophy.

Renal cortex mRNA expression

As previously described [23, 24] and summarized here, after the renal function analysis, one kidney was isolated, snap frozen and pulverized in liquid nitrogen, followed by the isolation of RNA using the TRIzol LS Reagent (Life Technologies, Carlsbad, USA). Then, 2 μ g total RNA was used to obtain cDNA (High-Capacity cDNA Reverse Transcription Kit; Life Technologies) and real-time PCR was performed using a StepOnePlus (Life Technologies) machine and TaqMan assay system (Life Technologies). The following TaqMan probes were used: angiotensinogen (*Agt*), Rn00593114_m1; renin (*Ren*), Rn00561847_m1; angiotensin I converting enzyme (*Ace*) Rn00561094_m1; NADPH oxidase isoform 4 (*Nox4*), Rn00585380_m1; NADPH oxidase subunit p22^{phox} (*Cyba*) Rn00577357_m1; eukaryotic translation initiation factor alpha (*Eif2a*), Rn01494813_m1; activating transcription factor 4 (*Atf4*), Rn00824644_g1; nuclear factor kappa B 1 (*Nfkb1*), Rn01399572_m1; interleukin 1 beta (*Il1b*), Rn00580432_m1; Bcl2-associated x protein (*Bax*), Rn02532082_g1; Bcl-2 (*Bcl2*), Rn99999125_g1 and the reference gene glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*), Rn01775763_g1. All qPCRs were performed using 20 ng cDNA, and all samples were assayed in duplicate. The comparative cycle threshold ($2^{-\Delta\Delta Ct}$) method was used for data analysis. The data were normalized to *Gapdh* expression and expressed as the fold change relative to the control group.

Immunohistochemical staining

As previously described [25] and summarized here, kidney sections (4- μ m thick) were deparaffinized and nonspecific protein binding was blocked by incubation with 10% goat serum in PBS for 60 min. Then, kidney sections were incubated overnight at 4°C with specific primary antibodies [rabbit polyclonal to renin (1:200, Biorbyt, San Francisco, CA, USA), mouse polyclonal to angiotensinogen (1:200, Biorbyt); rabbit angiotensin converting enzyme 1 (1:75, Novus Biologicals, Littleton, CO); rabbit NADPH oxidase 4 (1:200, Abcam, Cambridge, MA); rabbit eIF2 alpha (1:150, Cell Signaling, Denver, MA); rabbit I κ B alpha (1:100, Abcam); rabbit NF κ B1 p65 (1:75, Santa Cruz Biotechnology, Dallas, TX, USA); rabbit IL-1 beta (1:100, Santa Cruz Biotechnology); rabbit Bax (1:75, Santa Cruz Biotechnology), rabbit Bcl-2 (1:75, Cell Signaling, Denver, MA, USA) and subsequently with specific anti-rabbit or anti-mouse secondary antibodies for two hours at room temperature. The reaction products were detected using the avidin-biotin-peroxidase method (Vector Labs, Burlingame, USA) and the sections were counterstained with methyl green (Amresco, Solon OH, USA), dehydrated and mounted with Permount (Fischer Scientific, Fair Lawn, USA). Immunostained proteins were analyzed blindly by one independent person using a computerized morphometry program (NIS-Elements, Nikon). Positive cortical tubules for protein expression were counted in 20 fields (107172, 99 μ m²) and the mean count was calculated per kidney.

Statistical analysis

The data were evaluated using Student's t-test or non parametric test for comparisons between two groups depending on the sampling distribution, using GraphPad Prism Software (GraphPad Software, Inc, San Diego, CA, USA). The results are expressed as the mean $\pm$ standard error of the mean (SE) and p<0.05 was considered significant.

Results

Distribution of β -ARs in the kidney

We began confirming the expression and distribution of the β -ARs in the rat kidney tissue (Fig. 1). The β 1-AR immunoreactivity was found mostly in the proximal tubules and collecting ducts, whereas β 2-AR immunoreactivity was also observed in the proximal nephron and, to a lesser extent, in the collecting duct cells.

Physiological parameters, ventricles and kidney weight

As shown in Table 1, eight days of ISO treatment did not change food and water intake, neither the body weight gain or kidney weight. However, ISO treatment induced significant increase of the ventricles weight to body weight ratio, indicating the effectiveness of ISO to induce cardiac hypertrophy.

Renal hemodynamics and glomerular area

Eight days of ISO treatment significantly decreased RPF compared with control rats [(ml/min) CTL, 6.13 ± 0.21 versus ISO, 5.22 ± 0.30] (Fig. 2 A). Despite reduced glomerular perfusion in ISO-treated rats, the GFR was similar to control group [(ml/min) CTL, 1.81 ± 0.11 versus ISO, 2.06 ± 0.12] (Fig. 2 B). Consistent with these findings, the FF (GFR/RPF ratio) was significantly increased in ISO-treated rats compared with the control [(%) CTL, $30.52 \pm$

Fig. 1. Representative photomicrographs of the renal tissue, illustrating cortical and medullar area from control rats. Kidney sections (4- μ m thick) were stained for β_1 and β_2 adrenergic receptors. Standard images were captured using a morphometric program (NIS-Elements, Nikon) with a $\times 20$ objective and also shown are magnifications of positive-stained proximal tubules (PT) and collecting ducts (CD). Bar = 50 μ m.

Table 1. Differences among experimental group. Values are mean $\pm$ SE, number of animals (n) per group is indicated in parenthesis. * $p < 0.001$ versus control (CTL); ISO, isoproterenol, GFR, glomerular filtration rate; FE, fractional excretion; P, plasma; U, urine; V, urine flow

Parameters	CTL (n = 7-10)	ISO (n = 8-12)
Food intake, g/day	19.7 ± 1.1	19.3 ± 0.9
Water intake, ml/day	35.1 ± 2.1	34.8 ± 1.9
Body weight gain, g/8 days	44.2 ± 6.3	44.7 ± 7.9
Final body weight, g	286 ± 7	297 ± 8
Ventricles weight/body weight, mg/g	2.64 ± 0.06	$3.66 \pm 0.11^*$
Kidney weight/body weight, mg/g	3.98 ± 0.05	3.76 ± 0.09
	CTL (n = 10-10)	ISO (n = 9-10)
Plasma Na^+ , mEq/L	139 ± 1	140 ± 2
Plasma osmolality, mOsmol/kg H_2O	292 ± 5	297 ± 5
	CTL (n = 9-10)	ISO (n = 7-12)
Urine flow, $\mu\text{L}/\text{min}$	13.06 ± 1.9	15.34 ± 1.48
Urine osmolality, mOsmol/kg H_2O	274 ± 28	262 ± 17
Urine protein-to-creatinine, mg/day	1.57 ± 0.13	1.34 ± 0.15
	CTL (n = 6-10)	ISO (n = 8-10)
Filtered load of Na^+ (GFR. P_{Na^+}), mEq/min	262 ± 12	284 ± 15
Excreted load ($\text{U}_{\text{Na}^+} \cdot \text{V}$), mEq/min	0.35 ± 0.50	0.37 ± 0.03
U_{Na^+} , mEq/L	29.57 ± 4.22	27.78 ± 2.62
FE Na^+ (%)	0.15 ± 0.04	0.15 ± 0.02
	CTL (n = 6)	ISO (n = 7)
Glomerular area, μm^2	3040 ± 62	2899 ± 27

0.62 versus ISO, 38.42 ± 1.30] (Fig. 2 C). However, the renal hemodynamic changes did not induce changes in the glomerular area (Table 1).

Tubular function, urine flow and proteins

As shown in Table 1, ISO treatment did not change the Na^+ plasma concentration, plasma or urine osmolality, urine flow or Na^+ tubular handling compared with the control group. In addition, ISO treatment did not change the urine protein concentration compared with control group.

Intrarenal mRNA and/or protein expression of oxidative stress and endoplasmic reticulum stress components

As shown in Fig. 3 and Table 2, ISO treatment induced significant increase in cortical mRNA expression of Nox 4 and p22^{phox} as well as eIF2 α and Atf4 when compared with the control rats (A and D). The immunostaining and semiquantitative analysis demonstrate that ISO treatment also induced significant increase of NOX4 and EIF2 α expression in the cortical tubular segments, including proximal tubules (B, C, E and F).

Intrarenal mRNA and/or protein expression of inflammatory components

The immunostaining and semiquantitative analysis demonstrate that ISO treatment induced significant increase in the cortical I κ B expression when compared with the control group (Fig. 4 A, B and Table 2), ISO treatment also induced increase in both mRNA and immunostaining of NF κ B1 in comparison to the control group (Fig. 4 C - E and Table 2). ISO treatment did not change the mRNA expression of IL-1 β ($p = 0.152$), but it induced a significant increase in the cortical immunostaining of this cytokine when compared with the control group (Fig. 5 A - C).

Fig. 2. Effects of ISO treatment on renal hemodynamics, including renal plasma flow (RPF, Panel A), glomerular filtration rate (GFR, Panel B) and filtration fraction (FF, Panel C). Values are means $\pm$ SE, ($n = 8$).

Table 2. mRNA and/or protein expression among experimental group. Values are mean $\pm$ SE, number of animals (n) per group is indicated in parenthesis. * $p < 0.001$, & $p < 0.01$, $^{\$}p < 0.01$, $^+p < 0.05$ versus control (CTL); ISO, isoproterenol

Parameters (mRNA, fold-change)	CTL (n = 6-8)	ISO (n = 6-8)
Nox 4	1.01 $\pm$ 0.06	1.59 $\pm$ 0.21 ⁺
p22 ^{phox}	1.02 $\pm$ 0.08	1.51 $\pm$ 0.21 ⁺
eIF2 α	1.00 $\pm$ 0.05	1.49 $\pm$ 0.13 [§]
ATF4	1.00 $\pm$ 0.04	1.45 $\pm$ 0.13 [§]
NF κ B1	1.00 $\pm$ 0.04	1.30 $\pm$ 0.10 ⁺
Il-1 β	1.02 $\pm$ 0.07	1.74 $\pm$ 0.30
Bax	1.00 $\pm$ 0.04	1.50 $\pm$ 0.09 ⁺
Bcl-2	1.01 $\pm$ 0.05	1.07 $\pm$ 0.04
Bax/Bcl-2 ratio	1.00 $\pm$ 0.06	1.32 $\pm$ 0.08 [§]
Agt	1.00 $\pm$ 0.04	1.42 $\pm$ 0.11 [§]
Renin	1.00 $\pm$ 0.02	1.69 $\pm$ 0.28
ACE1	1.01 $\pm$ 0.05	1.46 $\pm$ 0.15 ⁺
Parameters (Protein expression)	CTL (n = 4-6)	ISO (n = 3-6)
Nox 4	72.29 $\pm$ 8.75	124.70 $\pm$ 10.80 [§]
eIF2 α	19.56 $\pm$ 1.02	23.27 $\pm$ 1.12 ⁺
I κ B	19.01 $\pm$ 1.15	23.98 $\pm$ 0.78 [§]
NF κ B1	19.14 $\pm$ 0.99	22.41 $\pm$ 0.62 ⁺
Il-1 β	0.80 $\pm$ 0.08	3.2 $\pm$ 0.39 ⁺
Bax	10.12 $\pm$ 0.14	15.01 $\pm$ 0.70 ⁺
Bcl-2	10.04 $\pm$ 1.09	8.85 $\pm$ 1.35
Bax/Bcl-2 ratio	1.08 $\pm$ 0.14	1.75 $\pm$ 0.32
Agt	17.66 $\pm$ 1.46	20.02 $\pm$ 1.80
ACE1	12.90 $\pm$ 1.31	19.40 $\pm$ 1.91 ⁺
Cortical renin	17.83 $\pm$ 2.07	19.44 $\pm$ 1.49
Medullar renin	34.33 $\pm$ 7.21	83.54 $\pm$ 12.37 ⁺

Fig. 3. Quantitative PCR analysis of renal cortex from the control or ISO treated rats. The mRNA expression levels of Nox4, p22^{phox} (A), eIF2 α and ATF4 (D). Representative photomicrographs of the renal cortex, illustrating immunostaining and semiquantitative analysis of the Nox 4 (B and C), and Eif2 α (E and F) from control and ISO treated rats. Kidney sections (4- μ m thick) were stained using immunohistochemical method. Standard images were captured using a morphometric program (NIS-Element) with a $\times 20$ objective. Also shown magnifications of positive-stained proximal tubules (PT) for NOX 4 and EIF2 α from control and ISO treated rats. Values are means $\pm$ SE, (n = 6-8). All qPCR samples were assayed in duplicate.

Fig. 4. Representative photomicrographs of the renal cortex, illustrating immunostaining and semiquantitative analysis of I κ B (A and B) from control and ISO treated rats. The mRNA expression levels of Nf κ B1 and representative photomicrographs of the renal cortex, illustrating immunostaining and semiquantitative analysis of Nf κ B1 also are shown (C - E). Kidney sections (4- μ m thick) were stained using immunohistochemical method. Standard images were captured

using a morphometric program (NIS-Element) with a x 20 objective. Also shown magnifications of positive-stained proximal and distal tubules for I κ B and Nf κ B1 from control and ISO treated rats. Values are means $\pm$ SE, (n = 4-6). PT, proximal tubule; DT, distal tubule.

Fig. 5. Quantitative PCR analysis of renal cortex from the control or ISO treated rats. The mRNA expression levels of IL-1 β (A). Representative photomicrographs of the renal cortex, illustrating immunostaining and semiquantitative analysis of IL-1 β (B and C). Kidney sections (4- μ m thick) were stained using immunohistochemical method. Standard images were captured using a morphometric program (NIS-Element) with a x 20 objective.

Also shown magnifications of positive-stained proximal tubules (PT) for IL-1 β from control and ISO treated rats and negative control without primary antibody. Values are means $\pm$ SE, (n = 6-8). All qPCR samples were assayed in duplicate.

Fig. 6. Quantitative PCR analysis of renal cortex from the control or ISO treated rats. mRNA expression levels of Bax and Bcl-2 (A). Representative photomicrographs of the renal cortex, illustrating immunostaining and semiquantitative analysis of BAX and BCL-2 (B and C). Kidney sections (4- μ m thick) were stained using immunohistochemical method. Standard images were captured using a morphometric program (NIS-Element) with a x 20 objective. Also shown magnifications of positive-stained proximal and distal tubules for BAX and BCL-2 from control and ISO treated rats. Values are means $\pm$ SE (n = 6-8). All qPCR samples were assayed in duplicate. PT, proximal tubule; DT, distal tubule.

Intrarenal mRNA and/or protein expression of apoptotic components

As shown in Fig. 6 and Table 2, eight days of ISO treatment significantly increased the cortical mRNA expression of Bax compared with the control group. In contrast, the mRNA expression of Bcl-2 remained unchanged between groups. In this condition, the Bax/Bcl-2 ratio was significantly increased in ISO treated group when compared with the control group (A). The immunostaining and semiquantitative analysis confirmed the stimulatory effect of ISO on BAX content which exceeded BCL-2. However, the BAX/BCL-2 ratio was only slightly higher in the ISO-treated group when compared with the control (p = 0.06) (B and C).

Intrarenal mRNA and/or protein expression of RAS components

Our results showed that ISO treatment induced a significant increase in cortical mRNA expression of angiotensinogen and ACE1 but not renin (p = 0.28) when compared with the control group (Fig. 7 A and Table 2). The cortical ACE1, but not angiotensinogen (p = 0.33) or renin (p = 0.53) immunostaining was increased in ISO treated group when compared with the control group (Fig. 7 B - C and Table 2). In contrast, ISO treatment induced significant increase in medullar renin content when compared to control rats (Fig. 7 D, E and Table 2).

Discussion

Using isoproterenol as a non-selective β -ARs agonist, in the current study we evaluated *in vivo*, potential mechanisms underlying β -ARs overstimulation and the consequences of this stimulation on the early stages of renal injury.

Studies have revealed that seven days of the β -agonist isoproterenol treatment induces cardiac hypertrophy without changes in blood pressure parameters, but with a significant

Fig. 7. Quantitative PCR analysis of renal cortex from the control or ISO treated rats. The mRNA expression levels of angiotensinogen - Agt, renin - Ren, angiotensin I converting enzyme - ACE 1 (A) are shown. Representative photomicrographs of the renal cortex (B and C) or medulla (D and E), illustrating semiquantitative analysis of AGT, ACE1 and REN, and immunostaining of cortical ACE1 and medullar renin. Kidney sections (4- μ m thick) were stained using immunohistochemical method. Standard images were captured using a morphometric program (NIS-Element) with a x 20 objective. Also shown magnifications of positive-stained proximal tubules (PT) for angiotensinogen and ACE1 and collecting duct (CD) for renin from control and ISO treated rats. Values are means $\pm$ SE (n = 6-8). All qPCR samples were assayed in duplicate.

increase of extracellular matrix remodeling in heart and vascular wall [17, 26]. Our study corroborates these findings since we observed a pronounced ventricular hypertrophy with eight days of isoproterenol treatment.

Considering that we and others [4] observed that β -ARs are well distributed throughout the kidney and that there is little knowledge about the involvement of specific β -ARs stimulation in the progression of chronic kidney disease, we were encouraged to initially investigate whether the stimulation of these receptors by isoproterenol for eight days contributes to changes in renal hemodynamics and tubular function. Our study revealed that despite the absence of changes in glomerular morphology, the ISO treatment induced significant decrease in the RPF and a slight increase (13.3%) in the GFR, suggesting increase of both pre-and post-glomerular resistance. These changes led to the increase of FF, as a result of GFR and RPF ratio. Our observations with specific β -ARs stimulation are relevant since the renal vasoconstriction leads to renal ischemia and are in agreement with previous studies in which the hyperactivity of the SNS resulted in increased afferent and efferent arterioles resistance [27, 28].

Despite evidence on the positive correlation between sympathetic hyperactivity and progression of renal failure [7], in the current study, specific β -ARs stimulation by ISO treatment was not enough to induce changes in renal sodium handling or urine flow, suggesting maintenance of sodium balance. In addition, the ISO treatment did not change protein excretion. However, preserved tubular function is no guarantee of the absence of

early cellular events, which may progress to tubular injury.

Studies *in vivo* and *in vitro* have highlighted that in cardiomyocytes the β -AR stimulation can activate NAD(P)H oxidase to produce ROS and to induce ER stress, resulting in cellular damage [12, 29]. In the kidney, we observed that ISO treatment induced a significant increase of intrarenal genes encoding NAD(P)H oxidase Nox 4 isoform and p22^{phox} subunit. In addition, the increased immunostaining analysis confirmed the post-transcriptional effect of ISO on Nox4 mainly in the renal cortex.

Accumulating evidences suggest a crosstalk between the ROS generation and ER stress response. Indeed, Nox 4 can physically interact with the protein disulfide isomerase (PDI), which is involved with protein folding in the ER. In this condition, the ER homeostasis is changed and in the signaling cascade, activation of double-stranded RNA-activated protein kinase (PKR)-like endoplasmic reticulum kinase (PERK) leads to phosphorylation of the eukaryotic translation initiation factor alpha (eIF2 α) and activation of the transcription factor 4 (ATF4). ATF4, in turn, translocates to the nucleus and upregulates the expression of factors associated with apoptosis and organ injury [30]. In the current study, we observed that prolonged treatment with ISO resulted in significant increase of eIF2 α and Atf4 mRNA, as well as eIF2 α protein expression specially in tubular cells of renal cortex, suggesting early cellular events which lead to tubular injury.

Previous studies have suggested a cross talk between ROS and/or eIF2 α pathway and NF κ B activation under many stress conditions. It is known that eIF2 α can directly activate NF κ B [31]. Furthermore, in response to ER stress, the IRE1 α induces I κ B phosphorylation leading to nuclear translocation of NF κ B1, which promotes a variety of gene transcription, including inflammatory cytokines such as TNF- α and IL-1 and 6 in various tissues [32, 33]. Consistent with these observations, our results provided a link between ER stress/I κ B/NF κ B activation and inflammation confirmed by IL-1 β tubular immunostaining in the renal cortex of rats treated with ISO for eight days. Together these factors seem to be essential for the initiation and progression of renal injury.

Furthermore, it is important to note that oxidative stress, ER stress and inflammation have been highlighted as mechanisms involved in renal tubular cells apoptosis [34]. The Bax to Bcl-2 ratio indicates the susceptibility of a cell to apoptosis [35]. In the current study, we demonstrated that the treatment with ISO resulted in significant increase of Bax in both mRNA and protein levels, and it also increased the Bax/Bcl ratio in RNAm levels, suggesting that the β -adrenergic stimulation by ISO combined with intrarenal ROS, ER stress and inflammation could induce apoptosis in the cortical tubular cells and contribute to the progression of renal injury.

With reference to the intrarenal RAS, various studies demonstrated the renal tubular expression of angiotensinogen, renin and ACE1 [36-39]. *In vitro* studies have reported effect of ISO on gene expression of angiotensinogen [5, 9]. However, there is no evidence whether β -ARs stimulation by ISO changes the expression of these molecules *in vivo*. Our *in vivo* study revealed that ISO treatment induced increase of cortical mRNA expression of both angiotensinogen and ACE1. Furthermore, ISO treatment induced increase in the protein levels of cortical ACE1 and medullar renin, suggesting a potential contribution of ISO on intrarenal RAS stimulation.

Conclusion

The results of the current study showed that eight days of β -adrenergic stimulation with ISO induced changes in renal hemodynamic and increased intrarenal ROS production, ER stress, inflammation, apoptosis and RAS components expression. Taken together, our findings provide relevant information regarding the contribution of specific β -adrenergic hyperactivity on early stage of renal injury, suggesting that, in addition to cardiac hypertrophy, it may be also an important risk factor for the development of kidney diseases.

Disclosure Statement

The authors declare no conflicts of interest.

Acknowledgements

This work was supported by the Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP) to Maria Oliveira-Souza, Gerhard Malnic and Margarida de Mello-Aires (13/19569-3 and 13/23087-4, respectively), Fernando Augusto Malavazzi Casare (11/14022-0), Vanessa Gerolde Cardoso (14/19154-0), Karina Thieme (14/17251-9); and the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) to Mariana Charleaux de Ponte (grant number 14/151575-2). The authors thank Dr. Luciana Venturini Rossoni for all discussions.

References

- 1 Malpas SC: Sympathetic nervous system overactivity and its role in the development of cardiovascular disease. *Physiol Rev* 2010;90:513-557.
- 2 Cupples WA, Braam B: Assessment of renal autoregulation. *Am J Physiol Renal Physiol* 2007;292:F1105-1123.
- 3 Johns EJ, Kopp UC, DiBona GF: Neural control of renal function. *Compr Physiol* 2011;1:731-767.
- 4 Boivin V, Jahns R, Gambaryan S, Ness W, Boege F, Lohse MJ: Immunofluorescent imaging of beta 1- and beta 2-adrenergic receptors in rat kidney. *Kidney Int* 2001;59:515-531.
- 5 Chan JS, Wang TT, Zhang SL, Chen X, Carrière S: Catecholamines and angiotensinogen gene expression in kidney proximal tubular cells. *Mol Cell Biochem* 2000;212:73-79.
- 6 Zoccali C, Mallamaci F, Parlongo S, Cutrupi S, Benedetto FA, Tripepi G, Bonanno G, Rapisarda F, Fatuzzo P, Seminara G, Cataliotti A, Stancanelli B, Malatino LS, Cateliotti A: Plasma norepinephrine predicts survival and incident cardiovascular events in patients with end-stage renal disease. *Circulation* 2002;105:1354-1359.
- 7 Schlaich MP, Socratous F, Hennebry S, Eikelis N, Lambert EA, Straznicki N, Esler MD, Lambert GW: Sympathetic activation in chronic renal failure. *J Am Soc Nephrol* 2009;20:933-939.
- 8 Moniri NH, Daaka Y: Agonist-stimulated reactive oxygen species formation regulates beta2-adrenergic receptor signal transduction. *Biochem Pharmacol* 2007;74:64-73.
- 9 Wang TT, Wu XH, Zhang SL, Chan JS: Molecular mechanism(s) of action of norepinephrine on the expression of the angiotensinogen gene in opossum kidney cells. *Kidney Int* 1998;54:785-795.
- 10 Rüster C, Wolf G: Renin-angiotensin-aldosterone system and progression of renal disease. *J Am Soc Nephrol* 2006;17:2985-2991.
- 11 Benigni A, Cassis P, Remuzzi G: Angiotensin II revisited: new roles in inflammation, immunology and aging. *EMBO Mol Med* 2010;2:247-257.
- 12 Dalal S, Foster CR, Das BC, Singh M, Singh K: B-adrenergic receptor stimulation induces endoplasmic reticulum stress in adult cardiac myocytes: role in apoptosis. *Mol Cell Biochem* 2012;364:59-70.
- 13 Kao MP, Ang DS, Pall A, Struthers AD: Oxidative stress in renal dysfunction: mechanisms, clinical sequelae and therapeutic options. *J Hum Hypertens* 2010;24:1-8.
- 14 Brembilla-Perrot B, Muhanna I, Nippert M, Popovic B, Beurrier D, Houriez P, Terrier de la Chaise A, Claudon O, Louis P, Abdelaal A, State S, Andronache M, Suty-Selton C: Paradoxical effect of isoprenaline infusion. *Europace* 2005;7:621-627.
- 15 Ahmed R, Tanvir EM, Hossen MS, Afroz R, Ahmmed I, Rumpa NE, Paul S, Gan SH, Sulaiman SA, Khalil MI: Antioxidant Properties and Cardioprotective Mechanism of Malaysian Propolis in Rats. *Evid Based Complement Alternat Med* 2017;2017:5370545.

- 16 Nirmala C, Puvanakrishnan R: Protective role of curcumin against isoproterenol induced myocardial infarction in rats. *Mol Cell Biochem* 1996;159:85-93.
- 17 Davel AP, Fukuda LE, De Sá LL, Munhoz CD, Scavone C, Sanz-Rosa D, Cachoeiro V, Lahera V, Rossoni LV: Effects of isoproterenol treatment for 7 days on inflammatory mediators in the rat aorta. *Am J Physiol Heart Circ Physiol* 2008;295:H211-219.
- 18 Mohamed SS, Ahmed LA, Attia WA, Khattab MM: Nicorandil enhances the efficacy of mesenchymal stem cell therapy in isoproterenol-induced heart failure in rats. *Biochem Pharmacol* 2015;98:403-411.
- 19 Bidani AK, Griffin KA, Williamson G, Wang X, Loutzenhiser R: Protective importance of the myogenic response in the renal circulation. *Hypertension* 2009;54:393-398.
- 20 Emori T, Hirata Y, Ohta K, Kanno K, Eguchi S, Imai T, Shichiri M, Marumo F: Cellular mechanism of endothelin-1 release by angiotensin and vasopressin. *Hypertension* 1991;18:165-170.
- 21 Abdel-Sayed S, Nussberger J, Aubert JF, Gohlke P, Brunner HR, Brakch N: Measurement of plasma endothelin-1 in experimental hypertension and in healthy subjects. *Am J Hypertens* 2003;16:515-521.
- 22 Oliveira-Souza M, Malnic G, Mello-Aires M: Atrial natriuretic peptide impairs the stimulatory effect of angiotensin II on H⁺-ATPase. *Kidney Int* 2002;62:1693-1699.
- 23 Thieme K, Oliveira-Souza M: Renal hemodynamic and morphological changes after 7 and 28 days of leptin treatment: the participation of angiotensin II via the AT1 receptor. *PLoS One* 2015;10:e0122265.
- 24 Casare FA, Thieme K, Costa-Pessoa JM, Rossoni LV, Couto GK, Fernandes FB, Casarini DE, Oliveira-Souza M: Renovascular remodeling and renal injury after extended angiotensin II infusion. *Am J Physiol Renal Physiol* 2016;310:F1295-1307.
- 25 Shimizu MH, Volpini RA, de Bragança AC, Campos R, Canale D, Sanches TR, Andrade L, Seguro AC: N-acetylcysteine attenuates renal alterations induced by senescence in the rat. *Exp Gerontol* 2013;48:298-303.
- 26 Yokoyama T, Nakano M, Bednarczyk JL, McIntyre BW, Entman M, Mann DL: Tumor necrosis factor- α provokes a hypertrophic growth response in adult cardiac myocytes. *Circulation* 1997;95:1247-1252.
- 27 Kon V: Neural control of renal circulation. *Miner Electrolyte Metab* 1989;15:33-43.
- 28 DiBona GF, Kopp UC: Neural control of renal function. *Physiol Rev* 1997;77:75-197.
- 29 Fu YC, Chi CS, Yin SC, Hwang B, Chiu YT, Hsu SL: Norepinephrine induces apoptosis in neonatal rat cardiomyocytes through a reactive oxygen species-TNF α -caspase signaling pathway. *Cardiovasc Res* 2004;62:558-567.
- 30 Schönthal AH: Endoplasmic reticulum stress: its role in disease and novel prospects for therapy. *Scientifica (Cairo)* 2012;2012:857516.
- 31 Deng J, Lu PD, Zhang Y, Scheuner D, Kaufman RJ, Sonenberg N, Harding HP, Ron D: Translational repression mediates activation of nuclear factor kappa B by phosphorylated translation initiation factor 2. *Mol Cell Biol* 2004;24:10161-10168.
- 32 Guijarro C, Egido J: Transcription factor-kappa B (NF-kappa B) and renal disease. *Kidney Int* 2001;59:415-424.
- 33 Dobrovolskaia MA, Kozlov SV: Inflammation and cancer: when NF-kappaB amalgamates the perilous partnership. *Curr Cancer Drug Targets* 2005;5:325-344.
- 34 Taniguchi M, Yoshida H: Endoplasmic reticulum stress in kidney function and disease. *Curr Opin Nephrol Hypertens* 2015;24:345-350.
- 35 Oltvai ZN, Millman CL, Korsmeyer SJ: Bcl-2 heterodimerizes *in vivo* with a conserved homolog, Bax, that accelerates programmed cell death. *Cell* 1993;74:609-619.
- 36 Alhenc-Gelas F, Baussant T, Hubert C, Soubrier F, Corvol P: The angiotensin converting enzyme in the kidney. *J Hypertens Suppl* 1989;7:S9-S14.
- 37 Sibony M, Gasc JM, Soubrier F, Alhenc-Gelas F, Corvol P: Gene expression and tissue localization of the two isoforms of angiotensin I converting enzyme. *Hypertension* 1993;21:827-835.
- 38 Navar LG, Mitchell KD, Harrison-Bernard LM, Kobori H, Nishiyama A: Intrarenal angiotensin II levels in normal and hypertensive states. *J Renin Angiotensin Aldosterone Syst* 2001;2:S176-S184.
- 39 Kobori H, Harrison-Bernard LM, Navar LG: Expression of angiotensinogen mRNA and protein in angiotensin II-dependent hypertension. *J Am Soc Nephrol* 2001;12:431-439.