Original Paper

Cardiac Inflammation after Ischemia-Reperfusion of the Kidney: Role of the Sympathetic Nervous System and the Renin-Angiotensin System

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Abstract
Background/Aims: To investigate the role of the sympathetic nervous system (SNS) and renin-angiotensin system (RAS) in renal ischemia/reperfusion-induced (I/R) cardiac inflammatory profile. *Methods:* Left kidney ischemia was induced in male C57BL/6 mice for 60 min, followed by reperfusion for 12 days, and treatment with or without atenolol, losartan, or enalapril. The expression of vimentin in kidney and atrial natriuretic factor (ANF) in the heart has been investigated by RT-PCR. In cardiac tissue, levels of β_1-adrenoreceptors, adenylyl cyclase, cyclic AMP-dependent protein kinase (PKA), noradrenaline, adrenaline (components of SNS), type 1 angiotensin II receptors (AT$_1$R), angiotensinogen/Ang II and renin (components of RAS) have been measured by Western blotting and HPLC analysis. A panel of cytokines – tumour necrosis factor (TNF-α), interleukin IL-6, and interferon gamma (IFN-γ) – was selected as cardiac inflammatory markers. *Results:* Renal vimentin mRNA levels increased by >10 times in I/R mice, indicative of kidney injury. ANF, a marker of cardiac lesion, increased after renal I/R, the values being restored to the level of Sham group after atenolol or enalapril treatment. The cardiac inflammatory profile was confirmed by the marked increase in the levels of mRNAs of TNF-α, IL-6, and IFN-γ. Atenolol and losartan reversed the upregulation of TNF-α expression, whereas enalapril restored IL-6 levels to Sham levels; both atenolol and enalapril normalized

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IFN-γ levels. I/R mice showed upregulation of β_1-adrenoreceptors, adenylyl cyclase, PKA and noradrenaline. Renal I/R increased cardiac levels of AT_1R, which decreased after losartan or enalapril treatment. Renin expression also increased, with the upregulation returning to Sham levels after treatment with SNS and RAS blockers. Angiotensinogen/Ang II levels in heart were unaffected by renal I/R, but they were significantly decreased after treatment with losartan and enalapril, whereas increase in renin levels decreased. ***Conclusion:*** Renal I/R-induced cardiac inflammatory events provoked by the simultaneous upregulation of SNS and RAS in the heart, possibly underpin the mechanism involved in the development of cardiorenal syndrome.

Introduction

Close physiological and physio-pathological relationships between the kidney and heart are found that share several functions maintaining homeostasis [1]. Cardiorenal syndrome is characterized by different clinical conditions, with an overlap of cardiac and renal dysfunctions. One subtype of this pathology involves cardiac hypertrophy and failure after acute renal injury (AKI) [1]. AKI frequently leads to the development of chronic kidney disease, and may be associated with ischemia followed by reperfusion (I/R). Ischemia restricts renal blood flow, whereas reperfusion restores the circulation and O_2 supply [2]. Ischemic renal damage promotes the release of different inflammatory cytokines that mainly target the heart, and promote cardiovascular alterations following the involvement of the immune system [3].

Inflammation, a systemic condition mediated by multiple factors (cytokines, complement system, etc.) is a non-traditional risk factor in cardiovascular diseases. Recently, however, ischemia, infection and uremic milieu are known to stimulate several inflammatory components in both the kidney and heart [4, 5]. An association between inflammatory markers and outcomes has been documented in patients with chronic kidney disease [5-7]. Inflammation may, therefore, serve as a crucial link between increased cardiovascular risk and kidney disease. Nevertheless, the direct involvement of immune or inflammation mediated damage to the pathogenesis of cardiorenal syndromes requires confirmation [8].

An important role of interleukin-6 (IL-6) in the molecular mechanisms underlying the initiation of hypertrophy has been identified [9], a process involving the participation of the sympathetic nervous system (SNS). Moreover, endogenous catecholamines present in immune cells play a central role in the crosstalk between the SNS and immune system [10-14]. Trentin-Sonoda et al. [15] showed the participation of cytokines in cardiac lesions after renal I/R in mice. The evidence suggests an association between changes in the components of the renin-angiotensin system (RAS), levels of angiotensinogen II [16, 17], and cytokines [18], with increased cardiac mass and deleterious cardiac remodelling [19-21]. In the last 2 decades, the relationship between inflammatory cytokines, cardiac natriuretic factors, including atrial natriuretic factor (ANF) and acute cardiac lesions have been reported [22-25]. Of special interest is that important direct and indirect effects of RAS exist, likely mediated by local Ang II and baroreflex, respectively, which enhance SNS activity in the heart [16, 17].

We also found – with the use of transthoracic echocardiography, electrocardiogram and action potential recording in the left ventricle – that renal I/R provoked cardiac hypertrophy, increased cardiac wall, augmented myocyte width, and intense electric remodelling [15]. The leading hypothesis herein is that the systemic inflammatory response elicited by renal I/R [15] targets the heart and alters local RAS and SNS, underpinning the mechanism of a PKA-mediated dysregulation of the cardiac cytokine profile. Support for this hypothesis is the observation that the β-adrenergic stimulus increases a macrophage-mediated immune response in the presence of cAMP as the central mediator [26, 27]. With this hypothesis as a basis, we have investigated the responses to losartan (a specific antagonist of the Ang II type 1 receptors (AT_1R)) and enalapril (an inhibitor of the angiotensin converting enzyme (ACE)) to target RAS. Atenolol, a blocker of β_1-adrenoceptors (β_1-AR) was used to inhibit SNS.

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Materials and Methods

Animals

All surgical procedures and protocols abided by the Ethical Principles in Animal Research set forth by the Brazilian College of Animal Experimentation, and were approved by the Federal University of ABC Ethics Committee for Animal Research (029/2013 and 030/2013). Male C57BL/6 J mice, 8-week-old (22–28 g), were obtained from the São Paulo University, Institute of Biomedical Sciences (São Paulo, Brazil). Mice were housed in a temperature and light-controlled environment (24°C; 12/12-h light/dark cycle), and had free access to standard mice chow (Purina Agribands) and water until the experiments began.

Fig. 1. Flow chart showing chirurgical and pharmacological interventions in the Sham and I/R groups from the ischemia (or not) on day zero until sacrifice after 12 days of reperfusion (horizontal gray bar). Abbreviations: d.w, dissolved in water; i.p, intraperitoneal administration.

Table 1. Primer sequences used for gene expression analysis

Gene	Sense sequence	Antisense sequence
Cyclophilin A	AGCATACAGGTCCTGGCATC	AGCTGTCCACAGTCGGAAAT
Vimentin	AGATCGATGTGGACGTTTCC	TCCGGTACTCGTTTGACTCC
ANF	ATCTGCCCTCTTGAAAAGCA	ACACACCACAAGGGCTTAGG
TNF-α	ATGGCCTCCCTCTCATCAGT	CTTGGTGGTTTGCTACGACG
IL-6	CTCTGCAAGAGACTTCCATC	CTCTGTGAAGTCTCCTCTCCG
INF-γ	ACACTGCATCTTGGCTTTGC	CATGTCACCATCCTTTTGCCAG
β1-adrenergic receptor	TGCTCATCGTGGTGGGTAAC	ACCAGCAATCCCATGACCAG
Adenylyl cyclase	GGACAGATGCTCTCATCTTG	GGAGGAAGGAGACGGTGAG
PKA	AACATCTCGCCACCAGCTAC	GGAGAGTGGGAACCACTACG

Renal I/R protocol

Renal I/R followed a previously described protocol [15, 28]. Mice were anesthetized with an intraperitoneal injection of ketamine/xylazine (Syntec) (116 and 11.5 mg/kg, respectively). An abdominal incision was made, the left renal pedicle exposed and then occluded with a steel clamp (DL Micof). After 60 min, the steel clamp was removed, and reperfusion began that lasted 12 days. Sham-operated mice had only abdominal incision made, but the pedicle occlusion procedure was not given (Fig. 1).

Pharmacological treatments

SNS and RAS were blocked using the β_1-adrenoceptor (β_1-AR) blocker, atenolol (Sigma-Aldrich; 10 mg/kg intraperitoneal) [29] the AT_1R antagonist, losartan (Polydrug Laboratories; 10 mg/kg), and the ACE inhibitor, enalapril (Zhejiang Changming Pharmaceutical Co.; 40 mg/kg). Both RAS blockers were diluted in drinking water [30-32]. Losartan and enalapril were administered for 7 days and atenolol for 5 days until day 11 of reperfusion (Fig. 1). These time-points were chosen because the onset of elevated circulating cytokines and electrophysiological disturbances occurred at day 8 of reperfusion [15]. The doses of the β_1-AR blocker, the AT_1R antagonist, and the ACE inhibitor used were chosen from previous work with these drugs [29-32]. The animals (n = 84) were divided in the following 8 groups: (i) Sham; (ii) I/R; (iii) Sham treated with atenolol; (iv) I/R treated with atenolol; (v) Sham treated with losartan; (vi) I/R treated with losartan; (vii) Sham treated with enalapril; and (viii) I/R treated with enalapril. The experimental groups (Sham, I/R) and pharmacological treatments that they received are indicated on the *abscissae* of the figures.

Gene expression and protein abundance

RNA from heart ventricles extracted with Trizol® (Merck) was quantified using NanoDrop Lite™ spectrophotometer (Thermo Fisher Scientific©). Two μg of total RNA were reverse transcribed to obtain the cDNA used for real-time polymerase chain reaction (PCR) assays (Stratagene®, Mx3005P, Agilent Technologies). mRNAs levels for vimentin, ANF, β_1-AR, AC, PKA, renin, TNF-α, IL-6, and IFN-γ were analysed, the primer sequences being shown in Table 1. All the primers have been designed using web-based tools, which ensure high specificity with regard to the desired target (Spidey/Splign, Primer blast, Blast NCBI); we also used the melting curve as an indicator of single fragment amplification. The $2^{-\Delta\Delta CT}$ method was used to calculate changes among the groups. The corresponding figures show the fold changes relative to Sham values. The mean values obtained with Sham were taken as the unitary value, and the individual values from
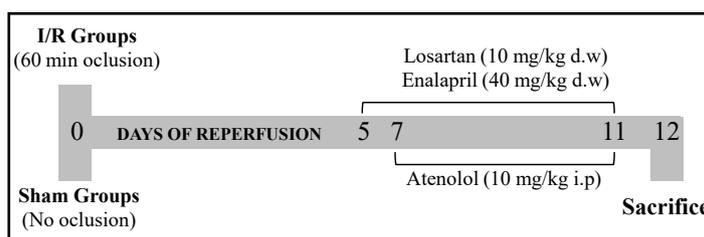
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the other groups were expressed as fold changes with respect to this value. This also allowed the S.D. of the different Sham values to be calculated.

Total heart ventricle protein was obtained using the radioimmunoprecipitation assay (RIPA) lysis buffer, its concentration being determined with a bicinchoninic acid kit (Thermo Fisher Scientific©). Aliquots of 40 µg proteins were resolved by electrophoresis (5% stacking/15% polyacrylamide

Table 2. List of primary and secondary antibodies used for protein content analysis. The manufacturer of secondary antibodies (rabbit and goat) was Jackson ImmunoResearch

Primary antibody	Titration of primary antibody	Titration of secondary antibody	Origin	Manufacturer primary antibodies	Catalogue number
Anti-GAPDH	1:5,000	1:10,000	Anti-rabbit	Santa Cruz Biotechnology	sc-47724
Anti-β-actin	1:1,1000	1:20,000	Anti-rabbit	Imuny Biotechnology	IM-0075
Anti-β$_1$-AR	1:250	1:10,000	Anti-rabbit	Thermo Scientific	PA1-049
Anti-adenylyl cyclase	1:500	1:20,000	Anti-rabbit	Thermo Scientific	PA1-31190
Anti-PKA	1:500	1:20,000	Anti-rabbit	Thermo Scientific	PA1-30236
Anti-AT$_1$R	1:1,000	1:1,000	Anti-rabbit	Santa Cruz Biotechnology	sc-579
Anti-angiotensinogen/Ang II	1:250	1:1,000	Anti-goat	Santa Cruz Biotechnology	sc-7419

sodium dodecyl sulphate [SDS] gel), before being transferred to nitrocellulose membranes (Bio-Rad®). The membranes were stained with Ponceau red solution and then washed with TBST (50 mM Tris, 150 mM NaCl, and 2% (w/v) Tween-20) for 10 min at 25–26°C. After overnight incubation at 4°C, the membranes were treated with peroxidase-conjugated secondary antibodies diluted in TBST for 1 h at room temperature. They were finally washed with TBST and incubated with enhanced chemiluminescence detection reagents (Thermo Fisher Scientific®), the chemiluminescent signals being detected using ChemiDoc (Bio-Rad®). Band intensity was measured by densitometry using ImageJ software. The antibodies are listed in Table 2. The Santa Cruz sc-7419 polyclonal antibody we used is considered as anti-angiotensin II (Ang II) according to the manufacturer [33, 34], but has been referred to as anti-angiotensinogen antibody by others [35, 36], as its molecular mass (~60 kDa) corresponds to that of angiotensinogen. We adopted dual denomination assuming that the levels of both molecules correlate well within cells.

Cardiac catecholamines

Catecholamines levels were determined in cardiac tissues by the method of Wojicyz et al. [37]. Adrenaline and noradrenaline data (in ng/mg wet cardiac tissue) were acquired in a high-performance liquid chromatography (HPLC) system (UltiMate 3000, Thermo Scientific™), according to Mishra et al. [38]. Calculations were carried out using adrenaline and noradrenaline standards (Y0000882 between 50 and 350 ng/mL, and N1100000 between 300 and 900 ng/mL, respectively), which gave rise to calibrated straight lines).

Analysis of PKA activity

The activity of PKA was assessed by quantification of the rate of incorporation of [γ-^{32}P]ATP γ-phosphoryl group (specific activity of approximately 1.5×10^{11} Bq/mmol) into histone in the absence or presence of PKAi, the specific inhibitor of PKA (final concentration 10 nM) [39-41]. The activity was expressed in pmol P~histone × mg^{-1} × min^{-1}).

Statistical analysis

Data are expressed as mean ± standard deviation (SD). The number of animals in each group is presented within the bars. Power analysis [42, 43] was used to determine sample size. Calculations were carried out according to Dell et al. [44] by using the equation $n = 1 + 2C \times (s/d)^2$, where C is the constant (7.85) for the levels of $\alpha = 0.05$ and statistical power $(1 - \beta) = 0.80$ [44]; and s and d are, respectively, the SD and difference expected in the comparisons between the untreated and the drug-treated animals from the Sham and I/R groups. The minimum was $n = 3$-4. The SD and the coefficients of variability intra- and inter-assay (<10 and <15%, respectively) were obtained in pilot studies.

Groups were compared using Student's *t*-test or one-factor analysis of variance (ANOVA), followed by Bonferroni test for selected pairs, as indicated in the figure legends. The significance levels have been indicated by the number of asterisks: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. Statistical analyses were carried out using GraphPad Prism 6 software (GraphPad Instruments Inc.).

Results

Renal vimentin mRNA levels after I/R

Real-time PCR was used on kidney samples to measure the gene expression level of vimentin (Fig. 2) as a known molecular marker of renal damage [45], which served for the characterization and validation of other markers [46, 47]. Renal vimentin mRNA levels that were barely detectable in the Sham group increased by 10-fold in I/R group (Fig. 2A), confirming severe renal impairment. Fig. 2B, C show the results from the experiments that investigated the effect of the 3 treatments on the expression of vimentin mRNA. Evaluation of the extent of lesion in Sham and I/R mice (Fig. 2B) showed that atenolol, losartan and enalapril

Fig. 2. Vimentin mRNA levels in the left kidney from the Sham (empty bars) and I/R (filled bars) C57BL/6J mice. Influence of treatment with atenolol, losartan, or enalapril. Data are expressed as mean ± SD. The number of experiments using different preparations is indicated within or above each bar. (A) Comparison of renal vimentin mRNA levels between Sham and I/R mice. Means were compared using Student's t-test; ****$p<0.0001$. (B) Effect of SNS and RAS blockade on renal vimentin mRNA level in Sham mice. Left side: atenolol; ****$p<0.0001$, as assessed using Student's t-test. Right side: losartan or enalapril, as indicated on the abscissa; *$p<0.05$, ***$p<0.001$, as assessed using one-factor ANOVA followed by Bonferroni's test for selected pairs. (C) Effect of blockade of SNS and RAS on renal vimentin mRNA level in I/R mice. Left side: atenolol; ****$p<0.0001$, as assessed using Student's t-test. Right side: losartan or enalapril, as indicated on the abscissa; ***$p<0.001$, as assessed using one-factor ANOVA followed by Bonferroni's test for selected pairs.

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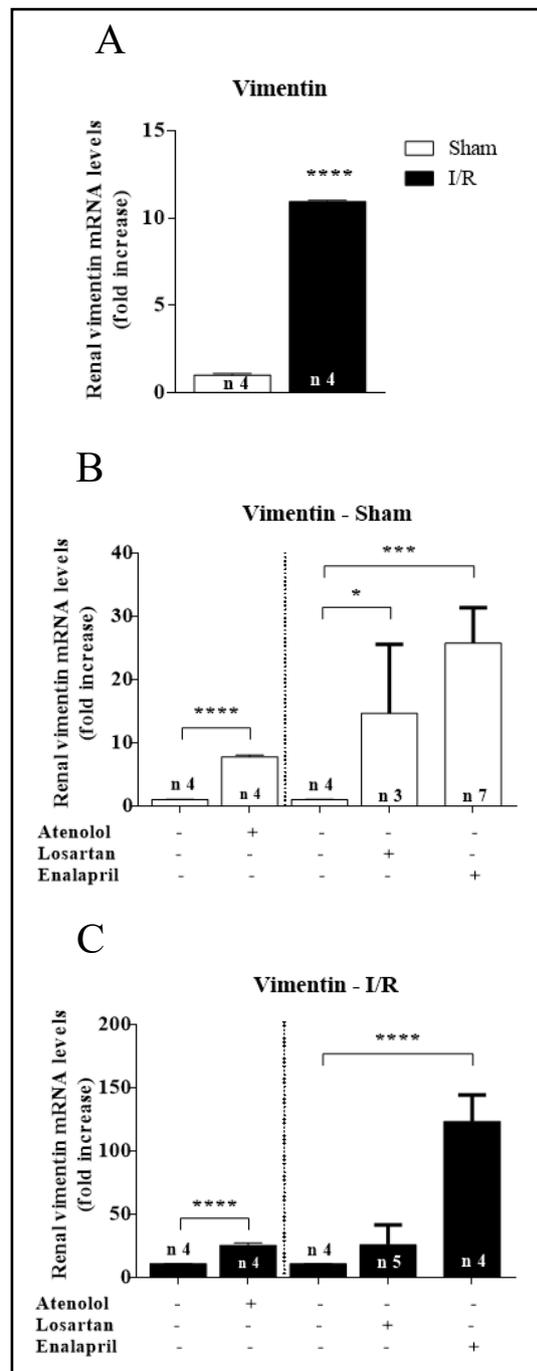Panico et al.: Cardiac Inflammation After Renal Ischemia

Fig. 3. Cardiac atrial natriuretic factor (ANF) mRNA levels of Sham (empty bars) and I/R (filled bars) mice. Influence of treatment with atenolol (A), losartan, or enalapril (B). Data are expressed as mean ± SD. The number of experiments using different preparations is indicated within each bar. Mice from each group were treated with or without drug, as indicated on the abscissae. **p<0.01, ***p<0.001, ****p<0.0001, as assessed by one-factor ANOVA followed by Bonferroni's test for selected pairs.
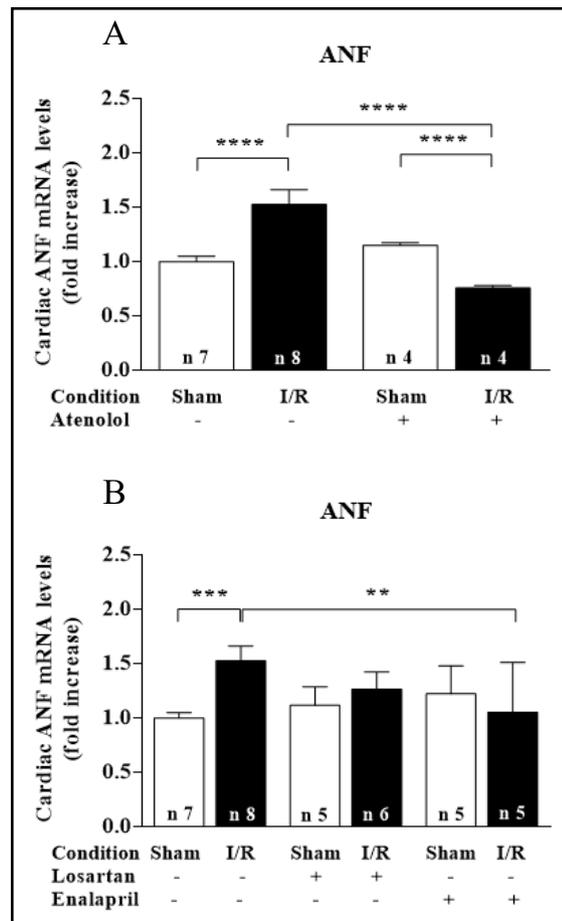
treatment increased vimentin expression 8, 14, and 25 times, respectively, compared with the sham group. Measurement of I/R (Fig. 2C) showed that atenolol and enalapril worsened the severity of the lesion (Fig. 2A). Vimentin expression was twice as high in mice treated with atenolol and 10 times in those receiving enalapril compared with untreated animals. Losartan treatment decreased the marked increase in the level of vimentin after enalapril treatment (Fig. 2C, right part).

Cardiac levels of ANF

ANF mRNA levels increased in the heart after renal I/R, this returning to the level below that observed for Sham group after atenolol treatment (Fig. 3A). No difference in ANF mRNA l was found between untreated and treated mice receiving losartan or enalapril (Fig. 3B). Enalapril decreased the mRNA level of ANF in the I/R group to that of the Sham group. The data strongly suggest that β_1-AR, ACE and AT_1R are involved in renal I/R-induced cardiac injury, as evident from the upregulated expression of ANF.

Expression of β_1-AR, AC, and PKA in the heart after renal I/R

AC and PKA are downstream components of the β_1-AR-linked signalling pathway [48]. The severity of renal I/R increased with an increase in the mRNA and protein expression of β_1-AR and AC in the targeted heart. Atenolol treatment restored the mRNA levels of β_1-AR and AC to the levels below those in the Sham group (Fig. 4A–D). Furthermore, AC increased in the heart samples from I/R group, but remained unaffected by atenolol treatment. The upregulated mRNA expression and increase in the level and activity of PKA α-catalytic subunit in renal I/R is shown in Fig. 5A–C. In Sham mice, atenolol treatment increased mRNA
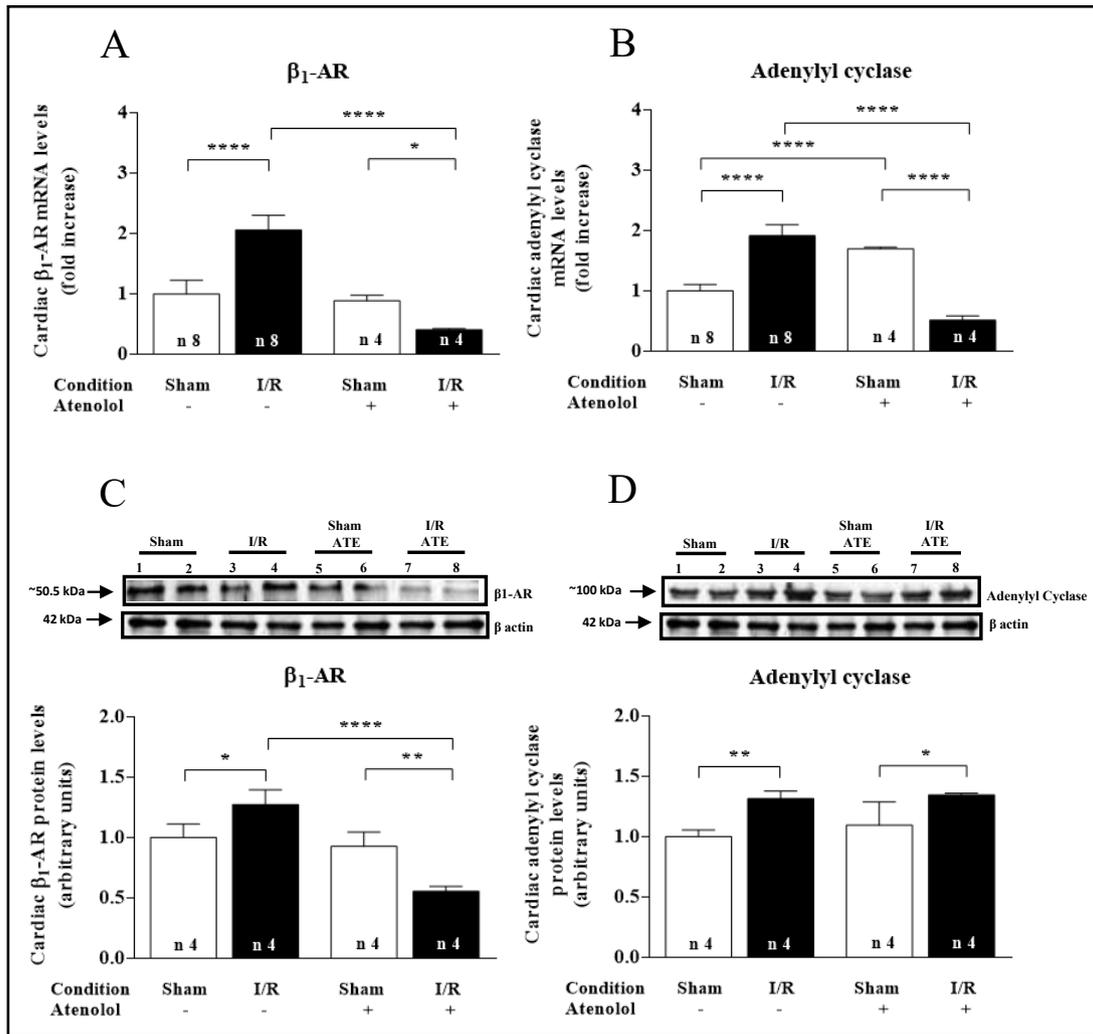
Fig. 4. Cardiac levels of β_1-adrenoreceptors and adenylyl cyclase (mRNA expression and protein abundance) after renal I/R. Data are expressed as mean ± SD. The number of experiments using different preparations is indicated within each bar. Mice from the Sham (empty bars) and the I/R (filled bars) groups were treated with or without atenolol, as indicated on the abscissae. (A) β_1-AR mRNA. (B) Adenylyl cyclase mRNA. (C) β_1-AR protein abundance. Upper panel: Representative immunodetection for β_1-AR and β-actin as control for protein loading. ATE: Treatment with atenolol. Lower panel: Graphical representation. (D) Adenylyl cyclase protein abundance. Upper panel: Representative immunodetection and β-actin as control for protein loading. Lower panel: Graphical representation. Differences were assessed using one-factor ANOVA followed by Bonferroni's test for selected pairs. *$p<0.05$, **$p<0.01$, ****$p<0.0001$.

(Fig. 5A) and protein (Fig. 5B) of PKA, but downregulated enzyme activity (Fig. 5C). In I/R, treatment with atenolol decreased PKA gene expression (Fig. 5A) and activity (Fig. 5C), with no effect on the protein level of PKA (Fig. 5B).

Fig. 5. Cardiac cAMP-dependent protein kinase (PKA). mRNA expression, protein abundance, and enzyme activity after renal I/R. Data are expressed as mean ± SD. The number of experiments using different preparations is indicated within each bar. Mice from Sham and I/R groups were treated with or without atenolol, as indicated on the abscissae. (A) PKA mRNA. (B) PKA catalytic -subunit abundance. Upper panel: Representative immunodetection for PKA and β-actin as control for protein abundance. ATE: Treatment with atenolol. Lower panel: Graphical representation. (C) PKA activity. *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$, as assessed using one-factor ANOVA followed by Bonferroni's test for selected pairs.

Noradrenaline Expression in Heart after Renal I/R

The levels of catecholamines in the heart tissues of Sham and I/R mice after 12 days of renal reperfusion were measured (Fig. 6). Noradrenaline was augmented in the I/R group compared to the Sham group in the absence of atenolol, and higher levels were in Sham and I/R mice treated with atenolol (Fig. 6A). No changes were observed in adrenaline (Fig. 6B).

Profile of different components of the cardiac renin-angiotensin system after renal I/R

Cardiac levels of AT_1R and angiotensinogen/Ang II are given in Fig. 7. The level of AT_1R increased by 30% in the I/R group than in the Sham group, and losartan and enalapril treatment had no effect on the Sham group. Both losartan and enalapril decreased significantly the level of the receptor in I/R mice (Fig. 7A). Angiotensinogen/Ang II levels were significantly lower in the receptor in the I/R group after treatment with receptor blocker and ACE inhibitor, whereas no effects were seen in the Sham animals (Fig. 7B).

Renal I/R significantly increased the expression of renin mRNA by >7-fold (Fig. 8). Treatments with atenolol, losartan and enalapril resulted in the restoration renin mRNA to normal values. Treatment of the Sham group with atenolol significantly increased the mRNA level of renin.
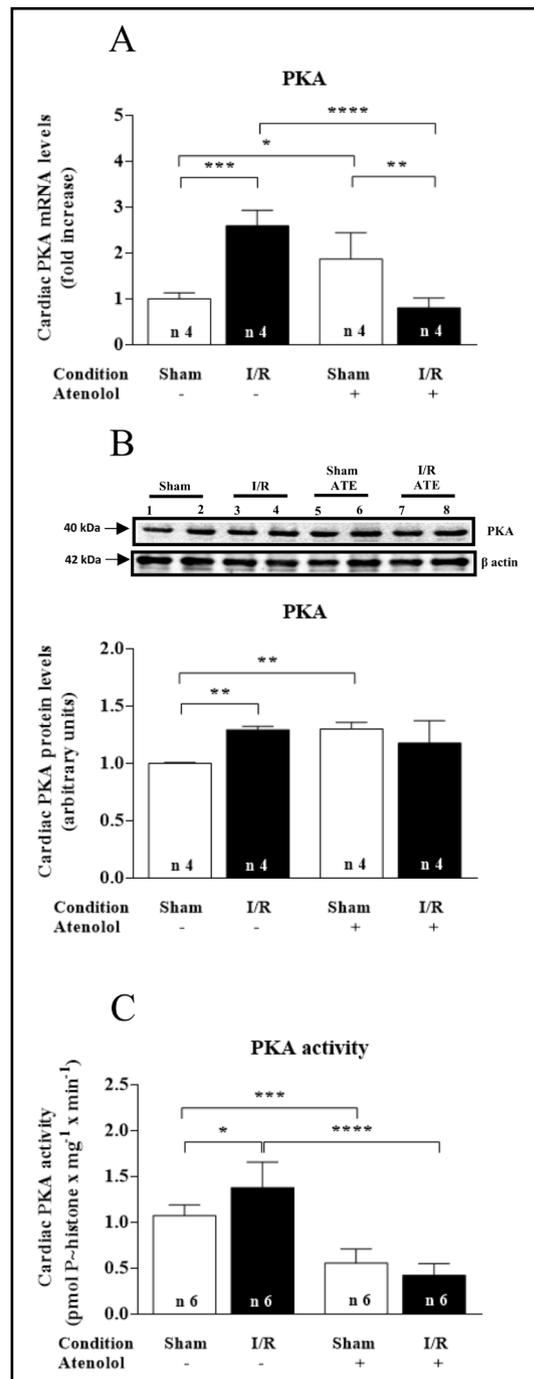
Fig. 6. Catecholamine levels in the heart tissue. Data are expressed as mean ± SD. The number of experiments using different preparations is indicated within each bar. Mice from Sham and I/R groups were treated with or without atenolol, as indicated on the abscissae. (A) Noradrenaline levels. (B) Adrenaline levels. ****P<0.0001 in A but no statistical differences in B, as assessed by one-factor ANOVA followed by Bonferroni's test for selected pairs.
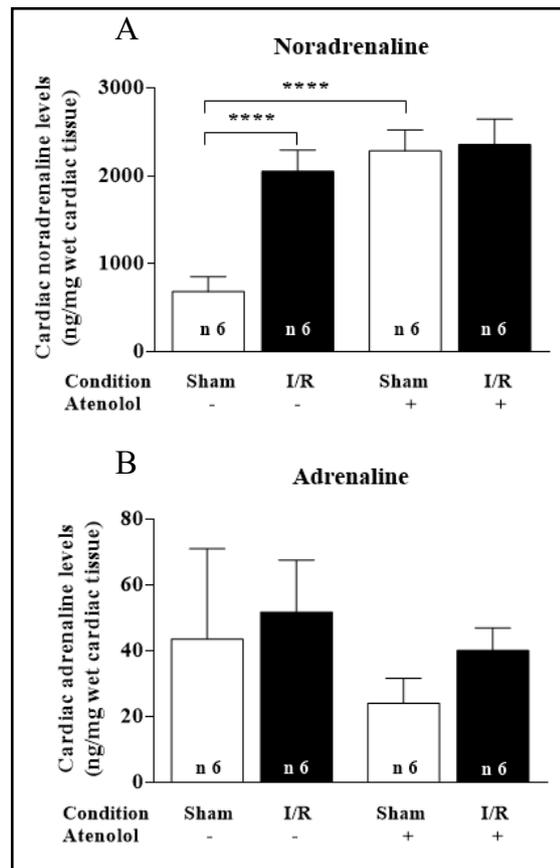
*Expression of inflammatory cytokines in the heart after renal I/R*

Fig. 9 shows the expression of the 3 key inflammatory cytokines in the heart tissue after renal I/R; in all there was a highly raised level:- 250% for TNF-α (Fig. 9A), 580% for IL-6 (Fig. 9B) and 470% for IFN-γ (Fig. 9C). The response of theses pharmacological treatments were different. In the case of TNF-α, the increase was reduced by atenolol and losartan, whereas the increase of IL-6 was attenuated only by enalapril. The augmented IFN-γ responded to atenolol and enalapril treatment.

Discussion

Renal I/R, the key pathophysiological event in AKI, is associated with high mortality rates [49, 50]. We have developed an experimental model to study the molecular mechanisms underlying SNS and RAS in cardiac lesions after renal I/R.

The damage induced by renal I/R was evident from increased mRNA expression of vimentin (Fig. 2), one of the most important molecular markers of renal damage [45], and other functional and structural markers, such as epidermal growth factor, proliferating cell antigen, c-Fos and clusterin [46, 47]. Trentin-Sonoda et al. [15] showed that: (i) I/R increased plasma levels of the markers of lesions, namely creatinine and urea, (ii) I/R induces renal parenchymal alterations detected by proton density magnetic resonance, as well as by signs of parenchymal edema. The renal lesion worsened following blockade of β1-AR with atenolol, indicating that renal SNS signalling is essential to prevent renal damage, an idea supported by the observation that the β-AR blocker induced vimentin expression in Sham mice. These puzzling observations about the kidney are suggestive of SNS-activated mechanisms similar to those beneficial ones described – or believed to occur – in ischemic preconditioning

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Fig. 7. Ang II type 1 receptor density and angiotensinogen/Ang II level in heart tissue after renal I/R. Data are expressed as mean ± SD. The number of experiments using different preparations is indicated within or above each bar. Mice from Sham (empty bars) and I/R (filled bars) groups were treated with or without different drugs, as indicated on the abscissae. (A) AT_1R abundance. Upper panel: Representative immunodetection for AT_1R and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as protein loading control. Lower panel: Graphical representation. (B) Angiotensinogen (AGT)/Ang II abundance and GAPDH as protein loading control. Upper panel: Representative immunodetection for AGT/Ang II and GAPDH as protein loading control. Lower panel: Graphical representation. **p<0.01, ***p<0.001, ****p<0.0001, as assessed using one-factor ANOVA followed by Bonferroni's test for selected pairs. Upper bands in A (a, b, c) and B (g, h, i) show for comparison 3 different representative immunodetections developed in separate experiments. Lower bands in A and B (d, e, f) show loading controls, which were the same for A and B.
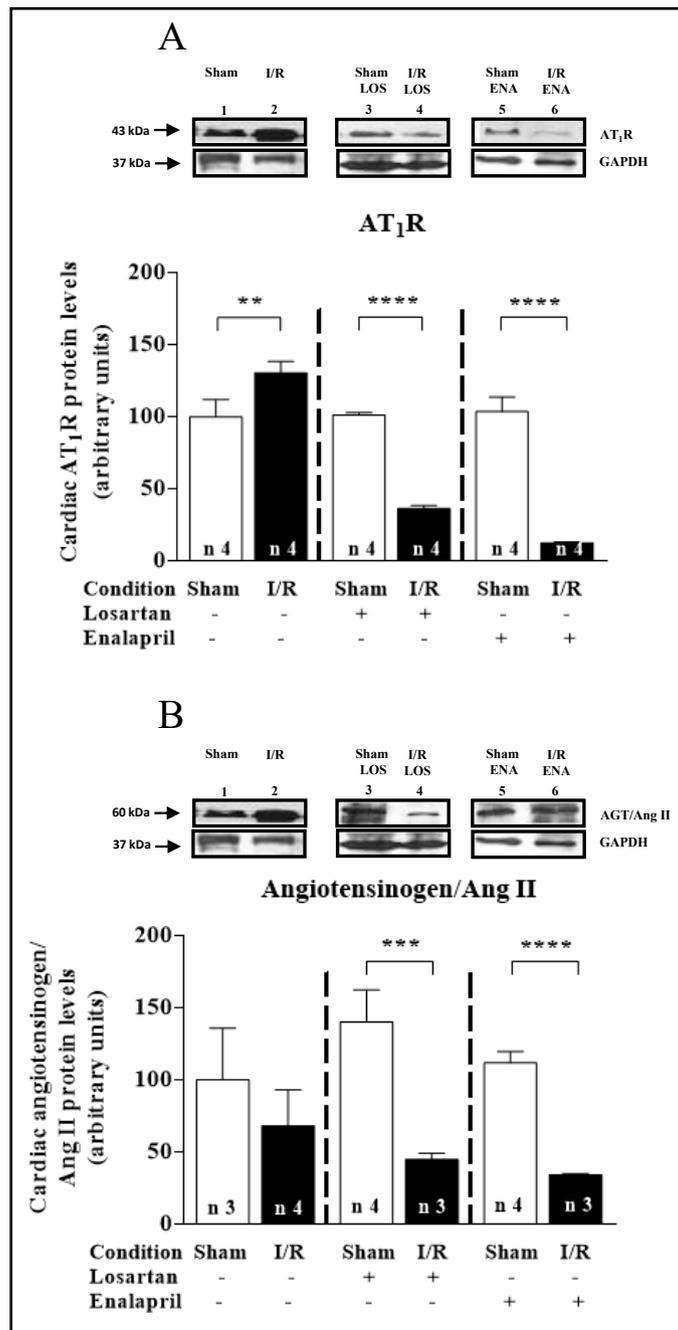
against I/R injury [51]. These effects elicited by SNS activation probably involve integrated central and peripheral mechanisms; they could increase local NO release [52], secretion of prostaglandins [53], or even increase perfusion pressure [54].

The results of the pharmacological manipulation of RAS suggest that Ang II formation is also important in minimizing, but not avoiding, ischemic lesion. As enalapril, but not losartan, increased the vimentin level, the signalling process downstream of renal AT_1R would not be altered in the injured kidney in the presence of adequate levels of Ang II. As no difference was found in plasma levels of Ang II between Sham and I/R mice (range 20–40 ng/mL), the integrity of local RAS (but not of systemic RAS) seems to be important in avoiding the worsening of I/R-induced renal lesions. The beneficial effects of Ang II are in line with those noted by Wenzel et al. [55]. Four possible mechanisms can be posited. First, Ang II

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Fig. 8. Renin mRNA levels in the heart tissue after renal I/R. Data are expressed as mean ± SD. The number of experiments using different preparations is indicated within or above each bar. Mice from Sham (empty bars) and I/R (filled bars) groups were treated with or without different drugs, as indicated on the abscissae. (A) Comparison of cardiac renin mRNA levels between Sham and I/R mice. Means were compared using Student's t-test; ****$p<0.0001$. (B) Effect of SNS and RAS blockade on cardiac renin mRNA level from Sham mice. Left side: atenolol; ***$p<0.001$, as assessed using Student's t-test. Right side: losartan or enalapril, as indicated on the abscissa; *$p<0.05$, as assessed using one-factor ANOVA followed by Bonferroni's test for selected pairs. (C) Effect of blockade of SNS and RAS on cardiac renin mRNA level in I/R mice. Left side: Atenolol; ****$p<0.0001$, as assessed using Student's t-test. Right side: Losartan or enalapril, as indicated on the abscissa; ****$p<0.0001$, as assessed using one-factor ANOVA followed by Bonferroni's test for selected pairs.
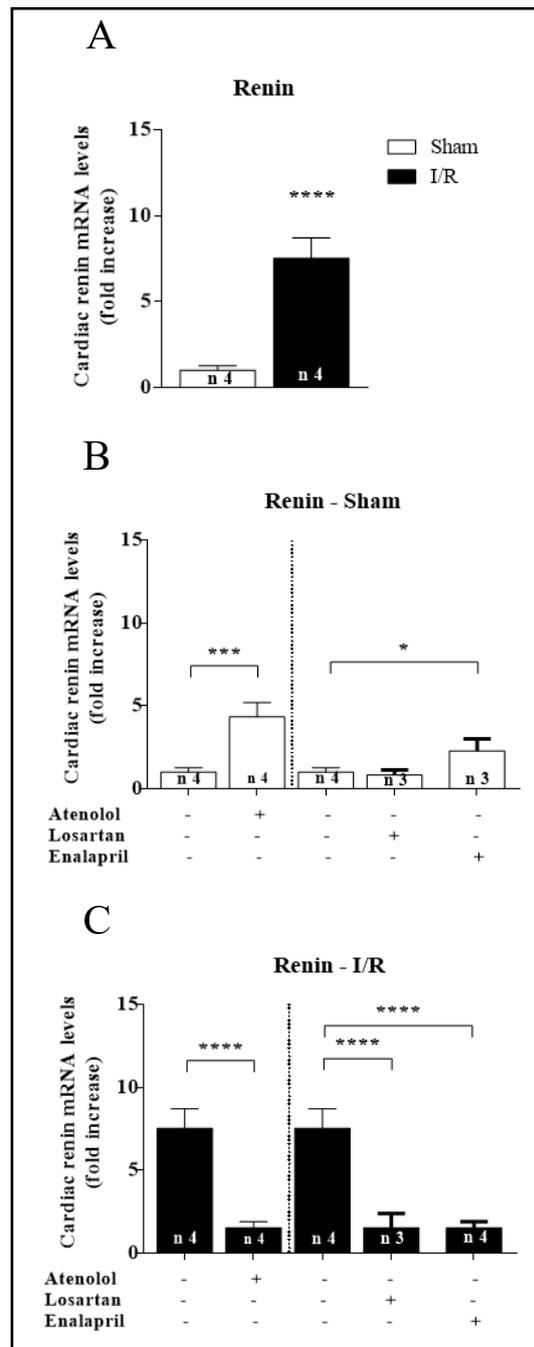
decreases the inflow of inflammatory mediators owing to its vasoconstrictor effects on renal blood flow, thereby reducing the inflow of disease activators [55]. Second, Ang II maintains endothelial nitric oxide levels in the renal tissue [56, 57]. Third, high local concentrations of Ang II activate the low-affinity AT_2R-linked signalling pathway, which is opposite to that of AT_1R [58]. Finally, a fourth mechanism could involve activation of the ACE2/Ang-(1–7)/Mas receptor axis, which protects against vascular and inflammatory injuries [59-62].

The scenario seems opposite when considering the molecular marker of cardiac injury ANF (Fig. 3). Inhibition of the increase in the ANF mRNA levels by atenolol, losartan and enalapril supports the view that both SNS and RAS are involved in renal I/R-induced cardiac lesion. Thus, blocking SNS or RAS may be beneficial for improving the structure and function of the injured heart. In a rat model of congestive heart failure after infarction, administration

Fig. 9. Inflammatory profile in cardiac tissue after renal I/R. Gene expression for TNF-α, IL-6, and IFN-γ was determined in Sham (empty bars) and I/R (filled bars) mice were treated with or without atenolol, losartan, or enalapril, as indicated on the abscissae. (A) TNF-α mRNA. (B) IL-6 mRNA. (C) IFN-γ mRNA. Data are mean ± SD. Differences were assessed using one-factor ANOVA followed by Bonferroni's test for selected pairs. *$p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.
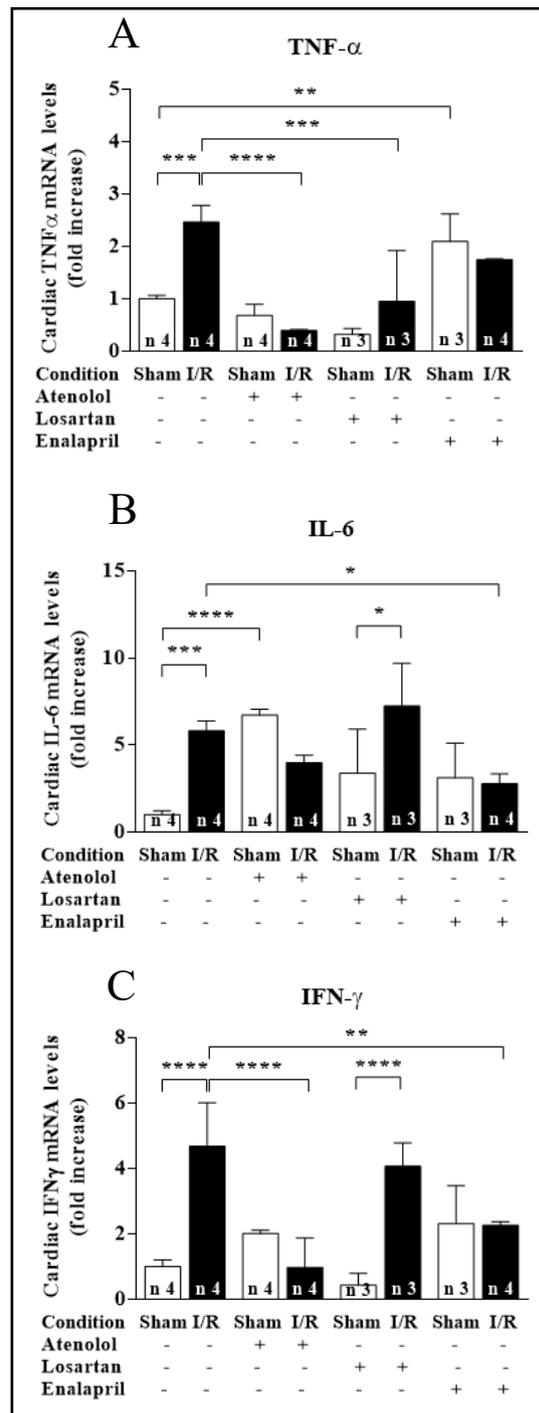
of atenolol or propranolol improved cardiac function and late myofibrillar remodelling [63]. RAS blocking likewise prevented myocardial I/R lesion and post-infarction remodelling [64]. The influence of atenolol and enalapril might be associated to the fact that local Ang II and sympathetic nerve activation stimulate ANP synthesis in myocytes. In a positive feedback situation, ANP stimulates RAS through signaling mechanisms involving mobilization of intracellular Ca^{2+} and activation of protein kinase C [65, 66]. Moreover, ANP, RAS inhibition and β-sympathetic nerve blocking modulate cytokine receptor expression and cytokine release in the heart as the result of the altered neurohumoral-immune cross-talk that occurs during cardiac remodeling (revised in [67]).

The high levels of noradrenaline in heart tissue ensure the recruitment of the agonist to high levels to maintain the permanent activation status of the pathway (Fig. 6). In addition, the fact that the noradrenaline, but not adrenaline, level increased without atenolol treatment may provide clues regarding the origin of the raised catecholamines. This observation seems to be a consequence of renal I/R-stimulated release of noradrenaline from the sympathetic nerve terminals in the cardiac tissue [68, 69], and not owing to the uptake from the plasma. During global SNS hyperactivation, the increase of adrenaline in the plasma arises from sympathoadrenal activation [70]. Renal I/R-induced cardiac adrenergic activation involves a transmitter that is more selective for β_1-AR than for β_2-AR [71], and may be the cause for hyperactivity in the pathway.

In terms of mechanisms, Fig. 4, 5 show the intriguing effects on the β_1-AR signalling pathway, which is selectively modified by renal I/R and can differentially respond to receptor blockade by atenolol. The renal I/R-induced increase in cardiac mRNA levels of β_1-AR, AC, and PKA is indicative of a global synchronous transcription stimulation encompassing the 3 components of the signalling cascade, which is abolished after treatment with the receptor blocker. Conversely, atenolol increased the mRNA levels of AC and PKA, which could be a normal physiological compensatory response in the case of decreased β_1-AR activity. Atenolol can upregulate or downregulate different genes [72], a property that could be different in Sham and I/R mice.

The abundance of the 3 proteins (β_1-AR, AC, and PKA) increased to about 30% in I/R mice compared to Sham mice, which shows the synchronous influence of renal lesion on the translation of the proteins associated with the cardiac β_1-AR signalling cascade. The fact that atenolol decreases the β_1-AR levels, but not the enzymes, suggests that the specific regulatory elements associated with these proteins in the heart are differentially activated/deactivated by renal I/R during the translation of their respective mRNAs. Several translational regulatory elements, with specific targets in physiological and pathological conditions, influence the number of protein molecules per mRNA molecule [73, 74].

The increased activity of SNS in the heart after renal I/R is accompanied with an upregulated expression of 2 components of the local RAS, as evident from the protein levels (AT$_1$R and angiotensinogen/Ang II) (Fig. 7) or mRNA levels (renin) (Fig. 8). An increase in cardiac AT$_1$R protein indicates that renal I/R injury upregulates the local cardiac RAS. The fact that the receptor density remained unmodified in the Sham mice and was low after losartan or enalapril treatment in I/R indicates that the actions of the drugs become evident in the presence of the humoral factors that are released following renal injury. One of these factors could be TNF-α, which is important in the progress of the I/R injury in mice [75], shown by the high levels in the plasma of our animal model [15]. The ~200% increase in TNF-α level in the heart several days after I/R supports this view. TNF-α might act by enhancing the rate of AT$_1$R transcription, as demonstrated in cardiac fibroblasts after post-myocardial infarction remodelling [76]. The same humoral I/R-induced environment seems to favour the similar effects of losartan and enalapril because the drugs decrease in parallel both RAS components.

Renal I/R-induced 6-fold increase in cardiac renin mRNA level (Fig. 8), which was restored to Sham levels after atenolol treatment – as also losartan and enalapril – is indicative of a regulatory effect of SNS on RAS signalling. Since cardiac angiotensinogen remained unmodified by renal I/R (Fig. 7), it is possible that renal I/R-induced upregulation of renin ensures the maintenance of Ang II levels and the short-term pathological structural modifications encountered in this model [15]. The increased level of renin in heart may be a consequence of the humoral signals resulting from renal I/R, or could be potentiated by activated SNS [77]. RAS stimulation results in the creation of a vicious circle that culminates in cardiac lesions in several pathologies [17]. The possibility that should also be considered is that increased cardiac renin results from increased binding and/or uptake of circulating prorenin and renin, which could be favoured by the same factors [78, 79].

Elevated cytokine levels in the plasma and distant organs, such as liver, lung [80], gut [81], brain [82] and other organs besides the heart [83], have been reported after renal I/R

(reviewed by [84]). The raised cardiac levels of TNF-α and IFN-γ (Fig. 9) matched those we previously reported in the plasma after 5 days of I/R [15], and increased expression of IL-6 occurs in the myocardium of subjects with cardiac dysfunction [85]. Therefore, these molecules may be considered as diverse and appropriate markers of inflammation of the heart in this renal I/R model, and their levels may increase in response to the local increase in β_1-adrenergic and RAS signalling [77, 86-88]. The relationship with β_1-AR-mediated signalling seems to be selective. Atenolol restores the levels of TNF-α and IFN-γ, but not IL-6; IL-6 levels were also shown to increase in Sham mice treated with blocker. These differences in the anti-inflammatory effect of atenolol could be attributed to a specific cytokine-modulated microenvironmental profile that modulates the sympathoimmune interactions in the tissue in different ways [88]. The differential responses of the 3 cytokines after losartan and enalapril treatment also point to the specific dependencies of each cytokine, with respect to different components of the RAS signalling cascade. High Ang II levels stimulate different arrays of pro-inflammatory cytokines in the kidney, leading to progressive renal injury [89]; a similar effect can be observed in the heart after renal I/R.

Finally, because blood pressure alterations after renal I/R could play a role as a connector of the renal-cardiac axis, we evaluated this parameter before and following I/R. Our studies did not reveal changes in systolic blood pressure even after the 12 days post-surgery. Indeed, blood pressure and heart rate post-surgery averaged 98 ± 5 mmHg and 584 ± 31 bpm in Sham animals ($n = 5$) and 99 ± 4 mmHg and 581 ± 21 bpm following I/R ($n = 3$) (P > 0.05 when each parameter was compared between the Sham and the I/R groups). These data indicate that the observed cardiac alterations are independent of blood pressure changes after renal I/R injury. Even though a late and sustained increase in losartan- and enalapril-sensitive blood pressure can occur at the early stages of heart injury after renal I/R, cardiac hypertrophy [15] is independent of blood pressure. Cardiac hypertrophy, however, has been frequently associated with hypertension in animals [90] and humans [91]. However, several reports also show that cardiomyocyte hypertrophy is independent of blood pressure in animals and humans in some cases [92-95]. Interestingly, a recent study has demonstrated that Ang II-induced hypertension and cardiac hypertrophy are differentially mediated by TLR3- and TLR4-signaling pathways [96], which are involved in renal I/R-induced cardiac damage [15]. It is possible that activation of these pathways is not synchronous, and for this reason, the onset of hypertension could be delayed.

Limitations of this Study
The pharmacological compounds used may have overlapping consequences and the targeted pathways could be mutually interacting, which is one limitation. The mechanism by which losartan and enalapril decrease AT_1R expression needs to be fully elucidated, though it could be explained by the consequence of an upstream decrease of renin and internalization/degradation of the inactivated receptor.

Conclusion

The results clearly demonstrate that both SNS and RAS have central roles in the mechanisms underlying upregulation in the expression of inflammatory cytokines in the heart after renal I/R. They support the proposition that renal I/R-induced cardiac inflammatory events provoked by the simultaneous upregulation of both systems in the heart are central in the mechanism involved in the development of a cardiorenal syndrome after a primary kidney lesion.

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Disclosure Statement

The authors declare no conflicts of interest.

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