Role of a RhoA/ROCK-Dependent Pathway on Renal Connexin43 Regulation in the Angiotensin II-Induced Renal Damage

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Abstract: In various models of chronic kidney disease, the amount and localization of Cx43 in the nephron is known to increase, but the intracellular pathways that regulate these changes have not been identified. Therefore, we proposed that: “In the model of renal damage induced by infusion of angiotensin II (AngII), a RhoA/ROCK-dependent pathway, is activated and regulates the abundance of renal Cx43”. In rats, we evaluated: 1) the time-point where the renal damage induced by AngII is no longer reversible; and 2) the involvement of a RhoA/ROCK-dependent pathway and its relationship with the amount of Cx43 in this irreversible stage. Systolic blood pressure (SBP) and renal function (urinary protein/urinary creatinine: Uprot/UCrea) were evaluated as systemic and organ outcomes, respectively. In kidney tissue, we also evaluated: 1) oxidative stress (amount of thiobarbituric acid reactive species), 2) inflammation (immunoperoxidase detection of the inflammatory markers ED-1 and IL-1β), 3) fibrosis (immune detection of type III collagen; Col III) and 4) activity of RhoA/ROCK (amount of phosphorylated MYPT1; p-MYPT1). The ratio Uprot/UCrea, SBP, oxidative stress, inflammation, amount of Cx43 and p-MYPT1 remained high 2 weeks after suspending AngII treatment in rats treated for 4 weeks with AngII. These responses were not observed in rats treated with AngII for less than 4 weeks, in which all measurements returned spontaneously close to the control values after suspending AngII treatment. Rats treated with AngII for 6 weeks and co-treated for the last 4 weeks with Fasudil, an inhibitor of ROCK, showed high SBP but did not present renal damage or increased amount of renal Cx43. Therefore, renal damage induced by AngII correlates with the activation of RhoA/ROCK and the increase in Cx43 amounts and can be prevented by inhibitors of this pathway.

Keywords: hypertensive nephropathy; oxidative stress; fibrosis; inflammation; Cx43; Fasudil

1. Introduction

Chronic kidney disease (CKD) is a worldwide public health problem whose prevalence is increasing. It is estimated that about 10% of adults in developed countries suffer some degree of kidney damage [1]. Patients with CKD usually develop a progressive kidney damage characterized by glomerular sclerosis and/or tubulointerstitial fibrosis, which eventually leads to end stage renal disease, which is the last stage of this condition [2]. The detrimental effect of this process includes the progressive reduction of the glomerular filtration rate (GFR) given by an increase in damaged nephrons, which eventually leads to organ failure [3]. CKD has different etiologies, including diabetic nephropathy, hypertensive nephrosclerosis and glomerulonephritis. However, regardless of the initial cause, the
Hypertension is one of the most common complications that predispose to other health problems and is the second cause of terminal CKD [6,7]. Hypertensive nephropathy starts in the glomeruli due to an increase in intra-glomerular pressure. These initial events activate and damage mesangial cells, epithelial cells and podocytes within the glomerulus. In turn, these cells produce vasoactive and pro-inflammatory mediators, which increase cell damage and favor fibrosis, reducing renal blood flow, permeability, and glomerular filtration [8]. The renin angiotensin system (RAS) is the prototype of a classic systemic endocrine network that acting on the kidneys and on the adrenal glands, regulate blood pressure, intravascular volume and electrolyte balance [9]. The vasoconstriction and increase in blood pressure mediated by angiotensin II (AngII) represent only part of the pleiotropic actions of this peptide. AngII stimulates aldosterone secretion, cell infiltration, proliferation and migration, thrombosis, superoxide ion production and other parameter altered in nephropathy [8]. In addition, high concentrations of AngII maintained for long periods of time, induce an inflammatory response characterized by the expression of pro-inflammatory cytokines such as interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) [10,11], infiltration of macrophages (ED-1 positive cells) [12], tubular overexpression of osteopontin (OPN) [12], and the expression of other pro-inflammatory cytokines, chemokines and adhesion molecules, which are ultimately associated with kidney damage induced by AngII [8,13]. Moreover, AngII increases the expression of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX), one of the main enzymes in the generation of reactive oxygen species (ROS), contributing to the onset of oxidative stress (OS), independent of the action of pro-inflammatory cytokines [14,15]. OS has been proposed to be involved in several pathological states such as cardiovascular diseases, infections, cancer, diabetes, neurodegenerative disorders and also in the progression of kidney damage [16]. Intrarenal RAS is an important factor in the pathophysiology of hypertension and hypertensive nephropathy [17]. Therefore, hypertension induced by the administration of AngII is a widely used model in the study of kidney damage, because in addition to providing a clinically relevant model of systemic hypertension, it produces progressive damage in the nephrons, which ultimately generates a chronic lesion, that eventually leads to end stage renal disease [8,17,18].

Two receptors, AT1 and AT2, both coupled to a G-protein, mediate the actions of AngII. The AT1 receptor activates small G proteins, including Ras, Rac1, RhoA and the Rho kinase system (ROCK) [19], while the AT2 receptor inhibits RhoA [20]. The Rho family of small GTPases (Rho GTPase) is constituted by monomeric G proteins between 20 and 40 kDa considered as molecular switches, which cycle between two conformational states, an active state bound to GDP and an inactive state bound to GTP, where RhoA is the most studied member of this family [21]. ROCK, a downstream effector of RhoA, is a serine-threonine kinase of around 160 kDa, which in mammals is present in two isoforms, ROCK1 and ROCK2 [22,23]. The RhoA/ROCK pathway plays an important role in renal pathophysiology, where RhoA/ROCK participates in the regulation of pro-inflammatory cytokines such as TNF-α and IL-1β [19,24], increases the amount of the transforming growth factor beta (TGF-β), and the nuclear factor-κB (NFκB) [22]. On the other hand, great interest has been generated in the use of Fasudil, a selective ROCK inhibitor as regulator in a wide variety of animal models of kidney damage, including unilateral ureteral obstruction, hypertensive glomerulosclerosis, acute renal failure induced by ischemia-reperfusion and renal failure induced by AngII [22]. Under experimental conditions, Fasudil has been shown to prevent kidney damage by preventing the increase in expression of extracellular matrix genes, OS, pro-inflammatory cytokines and macrophage infiltration, and inhibiting the cascade of events that leads to these effects. Thus, both RhoA and ROCK could be considered as therapeutic targets to prevent hypertension and kidney damage [19,22,24,25]. However, it remains unknown how to predict when kidney damage can be prevented along the development of a chronic condition, which would not only fulfill our intellectual interest, but also could serve as a relevant tool to improve the prediction of Fasudil treatment effectiveness in clinic.
The kidney regulates blood pressure mainly through excretion of Na\(^+\) and water, depending on the hormonal action of the RAS and other hormones with renal action \[26\]. However, in order to fulfill this function, the kidney requires the coordinated action of different cell types, including vascular and tubular cells \[26\]. The molecular mechanism of this intrarenal coordination has not yet been well established. A possible mechanism could involve cell-cell communication via gap junctions (GJs). In agreement, connexins (Cxs), protein subunits of gap junctions (GJs), are present in the kidney, and direct communication between the different cell types of the nephron has been proposed to occur through GJs and/or hemichannels (HCs) \[26\]. In this sense, 9 Cx subtypes have been detected in the kidney (Cx26, Cx30.3, Cx31, Cx32, Cx37, Cx40, Cx43, Cx45 and Cx46), which are located in the vasculature or in different segments of the renal tubule, where most likely fulfill different physiological functions \[26\].

In cortical astrocytes, it has been demonstrated, that two pro-inflammatory cytokines, TNF-\(\alpha\) and IL-1\(\beta\), reduce intercellular communication mediated by GJs and increase the permeability of the membrane through the HCs formed by Cx43 (Cx43 HCs) \[27\]. A similar change occurs in GJs and Cx43 HCs in astrocytes subjected to hypoxia-reoxygenation and in the presence of a high concentration of extracellular glucose \[28\] and in mesangial cells exposed to high glucose and pro-inflammatory cytokines \[29\]. This opposed regulation of GJs and Cx43 HCs by pro-inflammatory factors also occur in cultures of proximal tubule cells treated with metabolic inhibitors or pro-inflammatory cytokines, where an increase in the activity of Cx HCs has been demonstrated \[30,31\]. In pathological conditions such as hypertension, the amount of renal Cxs is altered. For example, in the two kidneys-one clip model, an increase in the amount of Cx43 mRNA and protein was observed \[32\]. In addition, the amount of Cx43 is increased in inflammatory processes, in damaged renal tubules and in interstitial cells in human kidneys \[33\]. In three different models of CKD (i.e., the transgenic renin [Ren\(^{+}\/+\)] model, the administration of antibody against the glomerular basement membrane [\(\alpha\)-GMB] and the unilateral obstructive uropathy), Toubas et al., observed an increase in the renal amounts of Cx43, so they postulated that the change in this Cx was caused by the development of inflammation in the damaged kidney \[34\]. Although renal tissue expresses several Cxs, only a few studies have described the involvement of GJs and HCs in kidney damage or in renal cell lines exposed to pathological condition and no signaling pathway has been clearly associated with these changes, but is know that the Cx43 is expressed in the renal parenchyma and the vasculature under resting and/or pathological conditions in both animals and humans and it is found de novo expressed and/or differentially localized in various renal cell populations during progression of renal disease, it would indicated an abnormal connexin signaling, both in patients and animal models \[30,31,34,35\].

Therefore, there role in normal renal tissue or in the development and progression of kidney damage is largely unknown. Based on the evidence described above, in this study we evaluated whether the RhoA/ROCK pathway is activated and regulates the abundance of Cx43, which might contribute to renal tissue damage in the model of renal damage induced by infusion of AngII.

2. Results

2.1. Four weeks of treatment with AngII causes an increase in blood pressure and decrease in renal function.

We first studied the time course of renal damage induced by AngII as well as the moment when the kidney stops recovering spontaneously from the damage induced by AngII. For this purpose, we evaluated the damage in kidneys 2 weeks after removing the infusion pump from animals treated for 2, 3 or 4 weeks with AngII, (Figure 1A). Systolic blood pressure (SBP) was elevated in rats treated with AngII for 2, 3, 4, 5 and 6 weeks (in mmHg; AngII 2 = 170.0 ± 13.4, AngII 3 = 159.0 ± 18.1, AngII 4 = 192.0 ± 18.0, AngII 5 = 217.0 ± 3.0 and AngII 6 = 191.0 ± 12.0, respectively) used as positive control groups. Also, in rats treated with AngII for 4 weeks, and measured 2 weeks after removal of the pump, SBP remained high (AngII 4+2 = 4 = 160.0 ± 16.0 mmHg). In contrast, SBP returned to normal values (in mmHg; Ctrl 4 = 103.0 ± 6.0, Ctrl 5 = 95.0 ± 4.0 and Ctrl 6 = 103.0 ± 8.0) 2 weeks after withdrawing...
the stimulus in rats treated for 2 and 3 weeks with AngII (in mmHg; AngII 2+2 = 100.0 ± 6.0 and AngII 3+2 = 99.0 ± 4.0) (Figure 2A).

To determine the degree of renal damage caused by the AngII treatment described above, the ratio urine protein/urine creatinine (UProt/UCrea) was measured. In rats treated with AngII for 2, 3, 4, 5 and 6 weeks this ratio was high (in arbitrary units, AU: AngII 2 = 20.6 ± 3.7, AngII 3 = 22.0 ± 7.9, AngII 4 = 50.0 ± 18.2, AngII 5 = 31.7 ± 10.2 and AngII 6 = 47.0 ± 5.4). However, in rats treated for 2 or 3 weeks and measured 2 weeks after stopping treatment with AngII, the ratio decreased (in AU: AngII 2+2 = 0.2 ± 0.1 and AngII 3+2 = 1.1 ± 0.1) to values similar to those of control rats (in AU: Ctrl 4 = 0.3 ± 0.2, Ctrl 5 = 0.3 ± 0.1 and Ctrl 6 = 0.4 ± 0.0). In contrast, in the group of rats treated with AngII for 4 weeks followed by 2 weeks without treatment, the ratio remained high (AngII 4+2 = 22.3 ± 9.5 AU) (Figure 2B).

Figure 1. Outlines of experimental protocols developed in male Sprague Dawley rats. (A) We used three experimental groups that were evaluated at 4, 5 and 6 weeks, respectively. Each group was divided into three subgroups: one control Ctrl; another with AngII infusion (160 ng/ml/day) during 4, 5 and 6 weeks (AngII 4, AngII 5 and AngII 6); and a third one in which animals were treated with AngII for 2, 3 and 4 weeks and then the treatment was suspended for the next 2 weeks (AngII 2+2, AngII 3+2 and AngII 4+2) (B) four experimental groups were used. These were divided into two control groups (Ctrl and Ctrl+Fasudil) and two groups that developed hypertension due to AngII administration for 6 weeks. At 2 weeks after the administration of AngII the systolic blood pressure (SBP) was measured and the groups were subdivided into rats treated with Fasudil (100 mg/kg/day) (AngII+Fasudil) for 4 weeks and rats that continued to be treated with only AngII until completing 6 weeks (AngII).
While the SBP remains elevated, the renal function diminished in rats after 2 weeks of withdrawal from 4 weeks treatment with AngII. SBP (mmHg) was measured by plethysmography (A). Protein and creatinine were measured in urine samples to assess renal function from ratio UProt/UCrea (B). The bars represent the means ± SE of a n≥4 rats per experimental group. The differences between the subgroups in each of the three groups were evaluated by an ANOVA followed by a Tuckey test. ***p<0.001, **p <0.01 and * p <0.05 vs AngII groups; &p<0.05 vs AngII 4+2.

2.2. The suspension of AngII infusion does not reduce OS, inflammation or renal tissue damage in rats infused with AngII for 4 weeks.

The basic pathophysiological mechanisms of renal disorders are associated with redox imbalance and inflammatory response [11,36]. Ischemic or toxic phenomena that can damage the tubules, as well as glomeruli can be accompanied by excessive generation of ROS, and pro-inflammatory cytokines such as IL-1β and TNF-α [10,11,36]. In addition, in a wide range of renal diseases, macrophage infiltration (ED-1 positive cells) is closely related to the upregulation of tubular expression of osteopontin (OPN). OPN is a potent chemoattractant expressed by damaged kidneys and acts as an adhesion molecule for monocytes and macrophages [12,37]. Also, the development of interstitial fibrosis is thought to be the cause of the irreversibility of renal dysfunction, since myofibroblasts (Alpha-smooth muscle actine, [α-SMA] and collagen type III [Col III] positive cells) in the damaged renal tissue are the main cell effectors of renal fibrosis [38].

OS estimated through the concentration of TBARS in the supernatant of renal homogenates samples from rats treated with AngII during 2, 3, 4 and 6 weeks (in μmol/g; AngII 2 = 2.9 ± 0.2, AngII 3 = 2.9 ± 0.4, AngII 4 = 2.3 ± 0.3, and AngII 6 = 3.8 ± 0.3) was significantly higher than in samples of control kidneys (in μmol/g; Ctrl 4 = 1.3 ± 0.2, Ctrl 5 = 2.0 ± 0.1 and Ctrl 6 = 2.4 ± 0.2). This increase was not observed in samples from rats treated with AngII for 5 weeks (2.3 ± 0.2 μmol/g). The amounts of TBARS were similar to those found in kidneys obtained 2 weeks after stopping the AngII treatment in rats treated for 2, 3 and 4 weeks with AngII (in μmol/g; AngII 2+2 = 1.3 ± 0.2; AngII 3+2 = 2.0 ± 0.3 and AngII 4+2 = 2.4 ± 0.2) (Figure 3A). In addition, when this same parameter was measured in plasma samples from rats treated with AngII during 2, 3, 4, 5 and 6 weeks the amount of TBARS was higher (in μmol/L; AngII 2 = 3.5 ± 0.3, AngII 3 = 6.1 ± 0.6, AngII 4 = 9.9 ± 1.4, AngII 5 = 19.0 ± 1.9 and AngII 6 = 20.0 ± 4.1) compared to the value detected in the plasma of the respective control groups (in μmol/L;
Ctrl 4 = 3.4 ± 0.4, Ctrl 5 = 10.0 ± 0.1 and Ctrl 6 = 4.7 ± 0.9). Two weeks after suspending the stimulus, the group of rats treated with AngII for 4 weeks still had high plasma TBARS concentration (AII 4+2 = 24.2 ± 3.8 μmol/L). However, 2 weeks after stopping the administration of AngII in rats treated for 2 and 3 weeks with AngII, the amount of TBARS in plasma decreased to values similar to those observed in control animals (in mmol/L; AngII 2+2 = 5.0 ± 1.5 and AII 3+2 = 6.6 ± 1.1) (Figure 3B).

The immunostaining for ED-1 showed a higher intensity in the group of animals treated for 4 weeks with AngII and was even greater in the groups treated for 5 and 6 weeks, compared with the control rats. After 2 weeks of AngII withdrawal in the rats treated for 2 and 3 weeks, the reactivity for ED-1 decreased, reaching an intensity similar to that of kidneys from control rats. In contrast, in kidneys from rats treated with AngII for 4 weeks, the reactivity for ED-1 was still high 2 weeks after AngII was suspended (Figure 4A). After 2 weeks of stopping the AngII treatment for 2 weeks, the amounts of IL-1β in plasmas decreased (AngII 2+2 = 206.0 ± 3.0 pg/g protein) to values similar to those found in the kidney of control rats (in pg/g protein; Ctrl 4 = 179.0 ± 20.0, Ctrl 5 = 185.0 ± 20.0 and Ctrl 6 = 203.0 ± 38.0). Interestingly, in kidneys from rats that were treated for 3 and 4 weeks with AngII, the amounts of IL-1β remained significantly elevated 2 weeks post-treatment with AngII (in pg/g protein; AngII 3+2 = 415.0 ± 26.0 and AngII 4+2 = 350.0 ± 12.0) (Figure 4B).

The degree of fibrosis, studied by immune detection of Col III, revealed more reactivity in kidney sections from rats treated with AngII during 2, 3, 4, 5 and 6 weeks, compared with that found in kidneys of control rats. However, 2 weeks after AngII withdrawal, the immunostaining for Col III decreased in kidneys of rats that had received 2 or 3 weeks of the AngII treatment to values similar to those found in kidneys of control rats, but the group treated for 4 weeks with AngII maintained high renal immunoreactivity for Col III 2 weeks after suspending the AngII administration (Figure 5).

**Figure 3.** The amount of TBARS remains high in the plasma but not in kidney of rats treated with AngII for 4 weeks. Supernatant of kidney homogenates (A) and plasma (B) were used to measure TBARS as marker of oxidative state. The bars represent the means ± SE of a n≥4 rats per experimental group. The differences between the subgroups of each of the three groups were evaluated by an ANOVA followed by a Tuckey test. ***p<0.001, **p <0.01 and * p <0.05 vs AngII groups; &p<0.05 vs AngII 4+2.
Figure 4. The amounts of ED-1 and IL-1 remain high in rats treated with AngII for 4 weeks. Immunostaining for ED-1 were performed in renal samples (A). Representative pictures of at least three different kidneys are shown. Scale bar = 100 μm. Brown staining represents ED-1 positive immunoreactivity. The staining is indicated by black arrows. The semi-quantitative morphometric analysis for ED-1 was performed as explained in Materials and Methods. Renal homogenates were used to measure IL-1 as marker of inflammation (B). The bars represent the means ± SE of a n=4 rats per experimental group. The differences between the subgroups of each of the three groups were evaluated by an ANOVA followed by a Tuckey test. **p <0.01 and * p <0.05 vs AngII groups; &&p<0.01 vs AngII 3+2 and AngII 4+2.
Figure 5. Col III remains high after 4 weeks of infusion with AngII. Immunostaining for Col III was performed in renal samples. Representative pictures of at least three different kidneys are shown. Scale bar = 100 μm. Brown staining represent the marker of Col III. The semi-quantitative morphometric analysis for Col III was performed as explained in Materials and Methods.

2.3. The increase in Cx43 and activation of RhoA/ROCK become independent of the stimulus after 4 weeks of treatment with AngII.

The phosphorylation state of renal myosin phosphatase target subunit 1 (MYPT-1), a downstream target of ROCK, in the group of rats treated with AngII during 4, 5 and 6 weeks was significantly higher (in AU: AngII 4 = 1.0 ± 0.1, AngII 5 = 0.9 ± 0.2 and AngII 6 = 1.1 ± 0.3) than in control rats. Two weeks after stopping treatment with AngII, the state of phosphorylation of MYPT-1 in kidneys from rats treated for 2 and 3 weeks with AngII (in AU: AngII 2+2 = 0.2 ± 0.0 and AngII 3+2 = 0.2 ± 0.0) was similar to control values (in AU: Ctrl 4 = 0.3 ± 0.1, Ctrl 5 = 0.2 ± 0.1 and Ctrl 6 = 0.2 ± 0.1). In contrast, in the kidney samples from the group of rats treated for 4 weeks with AngII, the phosphorylation status of MYPT-1 remained high 2 weeks after withdrawal of AngII administration (1.0 ± 0.1 AU) (Figure 6A).

Similarly, the amount of Cx43 was higher in the kidneys from rats treated with AngII during 4, 5 and 6 weeks (in AU: AngII 4 = 1.7 ± 0.1, AngII 5 = 1.6 ± 0.2 and AngII 6 = 1.3 ± 0.1) compared to the kidneys from control rats. Two weeks after stopping treatment with AngII, the amount of Cx43 in the kidneys of animals treated for 2 or 3 weeks with AngII decreased (in UA: All 2+2 = 0.3 ± 0.1 and AngII 3+2 = 0.4 ± 0.1) to values similar to those detected in kidneys from control rats (in AU: Ctrl 4 = 0.5 ± 0.1, Ctrl 5 = 0.3 ± 0.0 and Ctrl 6 = 0.5 ± 0.1). However, 2 weeks after stopping the treatment in rats treated with AngII for 4 weeks, the amount of Cx43 remained high (AngII 4+2 = 1.2 ± 0.1 AU) (Figure 6B).
Figure 6. The amount of phosphorylated MYPT and Cx43 remain elevated in rats treated with AngII for 4 weeks. Graphs show phosphorylation of MYPT-1 (A) and the relative amount of Cx43 (B). Under the graph representative pictures of phosphorylated MYPT (p-MYPT), unphosphorylated MYPT and Cx43 positive bands and their loading control (α-tubulin) are shown. The bars represent the means ± SE of a n≥4 rats per experimental group. The differences between the subgroups of each of the three groups were evaluated by an ANOVA followed by a Tuckey test. ***p<0.001, **p <0.01 vs AngII groups; &&p<0.01 vs AngII 4+2.

2.4. Fasudil prevents kidney damage, OS, inflammation, fibrosis, and the increase in protein amount of Cx43, but does not decrease SBP in rats treated with AngII.

After having identified 4 weeks of treatment with AngII as a point where renal damage is not spontaneously reversible, we evaluated the role of Rho/RACK on organism and organ parameters. For this purpose, we used four groups of animals; two control groups (Ctrl and Ctrl+Fasudil), and two experimental groups (AngII administered for 6 weeks and AngII+Fasudil, administered for the last 4 weeks of a 6 weeks treatment with AngII) were studied. Fasudil (100 mg/kg/day) was given in the drinking water (Figure 1B). The SBP in the rats treated only with AngII (192.0 ± 11.0 mmHg) and in rats treated with AngII plus Fasudil (AngII+Fasudil, 202.0 ± 10.0 mmHg) were significantly higher than those values recorded in their respective controls (Ctrl and Ctrl+Fasudil, in mmHg: 103.0 ± 8.0 and 97.0 ± 3.0, respectively). Therefore, Fasudil did not prevent the increase in SBP induced by AngII (Figure 7A).

The ratio U Prot/UCrea increased significantly in rats treated with AngII (73.0 ± 14.2 AU) compared with those of the control group (in AU: Ctrl = 0.4 ± 0.2, Ctrl+Fasudil = 0.4 ± 0.2). However, in rats treated with AngII+Fasudil, these values were similar to those of the control rats (1.9 ± 0.6 AU) (Figure 7B). In Table 1, the individual values for each parameter measured to assess the renal function...
of these rats are shown. A similar pattern was observed in proteinuria, fractional excretion of Na\(^+\) and K\(^+\) and creatinine clearance.

Consistent with previous results, the amount of TBARS (Figure 7C and D), the distribution of ED-1 (Figure 7E) and the amount of IL-1\(\beta\) (Figure 7F), Col III immunoreactivity (Fig. 7G), amounts of p-MYPT-1 (Figure 7H) and Cx43 (Figure 7I) in the group treated with AngII for 6 weeks were high, but in kidneys from rats treated with AngII+Fasudil the values for these parameters were similar to that detected in the kidneys of control rats.

Figure 7. Fasudil prevents kidney damage, OS, inflammation, fibrosis, and the increase in protein amount of Cx43, but does not decrease SBP in rats treated with AngII. (A). Protein and creatinine were measured in urine samples to assess renal function from ratio UProt/UCrea (B). Supernatants of kidney homogenates (C) and plasma (D) were used to measure TBARS as marker for the oxidative state. Immunostaining of ED-1 were performed in renal samples (E) and kidney homogenates were used to measure levels of II-1\(\alpha\) as marker for inflammation (F). Immunostaining of Col III were performed in renal samples (G). Representative pictures of at least three different kidneys are shown. Scale bar = 100 \(\mu\)m. Brown staining represents ED-1 and Col III positive immunoreactivity. The semi-
quantitative morphometric analysis for ED-1 and Col III was performed as explained in Materials and Methods. Graphs show relative amount of phosphorylated MYPT-1 (p-MYPT) (H) and the relative amount of Cx43 (I). Under the graph representative pictures of p-MYPT, unphosphorylated MYPT and Cx43 positive bands and its loading control (α-tubulin) are shown. The bars represent the means ± SE of a n>=4 rats per experimental group. The differences between the subgroups of each of the three groups were evaluated by an ANOVA followed by a Tuckey test. ***p<0.001, **p <0.01 and * p <0.05 vs AngII group.

3. Discussion

The incidence of CKD is increasing worldwide and there is no efficient therapy to reduce this phenomenon. The main therapies currently available focus on the control of blood pressure and the optimization of the blockade of the RAS [39]. In the present work, we addressed two questions. First, considering that AngII produces damage in the kidney, we identified the time point when renal damage turns irreversible, which resulted to be after 4 weeks of treatment with AngII since SBP, inflammation, OS, fibrosis, the amount of Cx43 and p-MYPT-1 remained high even after 2 weeks of AngII withdrawal. This could be explained by renal cell dysfunction that develops by the initial action of AngII and establishes interconnections between OS, inflammation, and fibrosis [40]. Although The half-life of bioactive peptides such as AngII is likely to be about a few hours [41], these interconnections coexist and communicate with each other independent of AngII, thereby exacerbating the processes underpinning these different entities with the end result of high morbidity and mortality in this model of kidney damage [40]. Also, we found an increase in kidney macrophage infiltration (ED-1) and renal amounts of IL-1β in response to AngII, indicating the presence of an inflammatory response mediated by the innate immune system. Interestingly, the amounts of IL-1β also remained elevated even when the stimulus was withdrawn. These findings suggest that kidney cells remain inflamed independent of the absence of cells of the innate immune system. This possibility is supported by the known activation of kidney inflammasome upon different injuries [42].

α-SMA and Col III also showed a similar response pattern. Accordingly, Ozawa et al. described that rats stimulated with AngII for 6 days and then 6 days without the stimulus, presented interstitial fibrosis, glomerular hypertrophy, macrophage infiltration, increase in MCP-1 and also in mRNA quantity of transforming growth factor-β (TGF-β) [8] showing a persistence of the effect even in the absence of the original stimulus. It is also known that TGF-β stimulates the synthesis of extracellular matrix and inhibits the action of proteases that degrade the matrix [9,43], favoring the development of fibrosis and inflammation in kidneys, finally reducing function and increasing kidney damage [9,12,44].

On the contrary, these parameters were spontaneously reversed in animals infused with AngII for 3 or less weeks, which indicates that AngII can generate alterations that can be compensated by kidney tissue that was not affected by AngII and/or can recover thanks to the small regeneration capacity of kidney tissue [45]. Spontaneous recovery has also been found by Frenay et al., who showed that newly formed renal lymph vessels can undergo spontaneous regression after cessation of AngII [45], suggesting that lymph vessel formation is a key event in renal interstitial fibrosis [46]. In the kidney, lymph angiogenesis occurs after proteinuria is established and prior to collagen deposition, fibrosis and macrophage infiltration, whereas angiotensin converting enzyme (ACE) inhibition significantly prevents this new lymph vessel formation [45,47]. Papakrivopoulos et al., by analyzing mouse models of irreversible and reversible glomerular injury, described that Van Gogh-like 2 (Vangl2), a protein that regulates planar cell polarity (PCP) is implicated in acquired kidney disease by modulating tissue remodeling that includes cytoskeletal rearrangements affecting cell morphology or changes in inflammation [48]. Indeed, downregulation of Vangl2 increased the activity of matrix metalloproteinase-9 (MMP9). MMP9 has previously been shown to be produced by podocytes and it is altered in a number of glomerular diseases, supporting the conclusion that Vangl2 modulates glomerular injury by stopping MMP9 production [48]. Therefore, it is possible that TGF-β, lymph angiogenesis and/or Vangl2 could be involved in the development of renal fibrosis and inflammation, thus it could be a key factor in the irreversibility of kidney damage; consequently,
additional studies are required to address and demonstrate the involvement of these factors in the reversibility/irreversibility of renal damage, independent of the initial stimulus.

We were also interested in predicting the effectiveness of Fasudil to reduce renal damage. For this reason, we directed our investigation to the RhoA/ROCK pathway. In this sense, the renal afferent arterioles are primarily responsible for regulating preglomerular resistance, renal blood flow, and GFR. Elevated renal vascular resistance and preglomerular reactivity are observed in AngII-induced hypertension [49]. Although many systemic, neural, paracrine, and autoregulatory mechanisms contribute to afferent arteriolar dynamics, in AngII-dependent hypertension a direct effect has been observed between the RhoA/ROCK pathway and the endogenous production of AngII [49]. We observed that, although treatment with Fasudil did not reduce SBP, the establishment of irreversible renal damage was prevented, reducing inflammation, OS, fibrosis, and also kept the amount of Cx43 and p-MYPT-1 at normal levels. Interestingly, the activity of the RhoA/ROCK pathway has been widely investigated in the pathogenesis of hypertension. Under this condition this pathway fulfill one of the important roles in regulating smooth muscle contraction, could lead to an increase in peripheral vascular resistance, which are characteristics observed in several hypertension models [50]. The protective effect of Fasudil in vivo is partly explained by its pleiotropic action in different settings; therefore, considering that ROCK inhibitors were developed as anti-hypertensive drugs, it is striking that in the present work Fasudil did not affect SBP, but did reduce the progression of kidney damage. Against the effect of this drug on blood pressure, several studies have established that Fasudil is renoprotective without affecting blood pressure, establishing a controversy regarding the use of Fasudil and its anti-hypertensive action [51–54]. In view of this, it is possible that tissues different from kidney take the lead in the hypertensive response. Similar interpretation might explain the persistent high TBARS concentration in plasma and evident recovery in kidneys function of mice subjected to the protocol AngII 4+2 (Figure 3B). Thus, the circulating TBARS in these mice might be generated in other tissues affected by the high SBP resistant to Fasudil. However, further studies remain to be done to elucidate why Fasudil does not prevent the increase in SBP, even when it prevents kidney damage.

In afferent arteriolar cells from rats treated with AngII the activation of NF-κB is mediated by the RhoA/ROCK pathway and the ROCK/NF-κB axis contributes to the upregulation of angiotensinogen, leading to an increase in the amount of intrarenal AngII [49]. In addition, in fibroblasts a direct relationship has been demonstrated between the activation of the RhoA/ROCK pathway and the increase in the amount of Cx43. In these cells, the expansion mechanisms in response to stretching involves the release of ATP to the extracellular medium through the RhoA/ROCK pathway and the increase in the amount of Cx43. In this work, AngII-induced alterations in cell membrane permeability could lead to activation of several metabolic pathways including the RhoA/ROCK signaling pathway by the progressive increase in the amount of p-MYPT in MES-13 cells, which promote OS and generation of pro-inflammatory cytokines [57]. Interestingly, we found similar changes in RhoA/ROCK activity and also found that ROCK inhibitors, prevented increases in the amount of Cx43 induced by AngII, indicating that activation of a RhoA/ROCK-dependent pathway and Cx43 HCs are regulated by the same transduction mechanism and intracellular signaling pathway activated by AngII. According to the results obtained in this work, we postulate that there is a direct correlation between the activity...
of a RhoA/ROCK-dependent pathway, Cx43 and renal damage in this model of hypertensive nephropathy.

In conclusion, the rat renal damage, inflammation, OS, changes in amount of Cx43 and activity of the RhoA/ROCK pathway are spontaneously reversible if the increase in AngII last 3 or less weeks. These alterations become irreversible and irreparable if AngII administration is maintained for 4 or more weeks. Fasudil, an inhibitor of the RhoA/ROCK pathway, prevents the irreversibility of the evaluated kidney alterations when is administered 2 weeks before the point of irreversibility revealing a close relationship between activation of a RhoA/ROCK-dependent pathway and increase in the amount of Cx43 in CKD (Figure 8). This would not only fulfill our intellectual interest but also could serve as a relevant tool to improve the prediction of Fasudil treatment effectiveness in CKD in a clinical context. The present results obtained with the least invasive measurements suggest that elevated SBP and UProt/UCrea with normal plasma TBARS would predict that Fasudil treatment might prevent the establishment of CKD induced by high AngII concentration.

4. Materials and Methods

4.1. Reagents and Antibodies

AngII was obtained from Alomone (Jerusalem, Israel). Fasudil, malondialdehyde tetrabutylammonium salt (MDA), phenylmethanesulfonyl fluoride (PMSF), Triton X-100, 3,3′-diaminobenzidine, λ-carrageenan, Tris-HCl, hydrogen peroxide, phosphate salts, and other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). The following primary antibodies were used: monoclonal antibody against macrophages (clone ED-1) was obtained from AbD Serotec (Kidlington, UK); antibody against collagen type III (Col III) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Secondary antibodies and the corresponding PAP complexes were purchased from ICN Pharmaceuticals-Cappel (Costa Mesa, CA, USA). The monoclonal anti-α-tubulin antibody was obtained from Sigma-Aldrich (St. Louis, MO, USA); the polyclonal anti-phosphorilated-MYPT1 (Thr696) antibody was obtained from Merck Millipore (Darmstadt,
Germany); the monoclonal anti-MYPT1 antibody was obtained from BD Transduction Laboratories (San José, CA, USA); the monoclonal anti-unphosphorylated Cx43 antibody was obtained from Invitrogen (Carlsbad, CA, USA); anti-mouse and anti-rabbit secondary antibodies conjugated to horseradish peroxidase were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA).

4.2. Animals

Adult male Sprague–Dawley rats (1-month-old, 100–120 g) were maintained under a 12 h light/12 h dark cycle, with food (20.5% protein, 5% fiber, 4% fat; Champion, Santiago, Chile) and water ad libitum at the university animal care facilities. All procedures were in accordance with institutional and international standards for the humane care and use of laboratory animals (Animal Welfare Assurance Publication A5427-01, Office for Protection from Research Risks, Division of Animal Welfare, NIH (National Institutes of Health), Bethesda, MD, USA), as described previously [58]. The experimental procedures were approved by the Bioethical and Biosafety Committee of the Faculty of Biological Sciences at the Pontificia Universidad Católica de Chile (CBB-064/2014).

4.3. AngII Administration and Experimental Procedure

We used the previously described hypertensive model caused by the prolonged administration of AngII [59]. Briefly, animals were anesthetized with a dose of ketamine/xylazine (25:2.5 mg/kg, respectively). A small (1 cm) cut was made in the mid scapular region and an osmotic pump was implanted (Alzet, Cupertino, CA, USA), and subsequently the wound was sutured. Pumps with a release rate of 160 ng/min per day for 6 weeks, were loaded with a high-dose of AngII dissolved in saline [59,60]. Pumps were kept in the animals for the duration of the experimental treatment. The Sham rats (controls) underwent the same surgical procedure, but without implanting the osmotic pump. After their recovery, the rats were maintained with water and food ad libitum. To check that the implanted rats did indeed develop high blood pressure, systolic blood pressure (SBP) was measured weekly. In the first set of experiments, we evaluated the spontaneous reversibility of the renal damage with AngII. For this purpose, rats were treated for 2, 3 or 4 weeks with AngII and then the pump was removed, and the animals were evaluated for 2 additional weeks later. As positive controls for these groups, rats kept for the whole period (2, 3, 4, 5 or 6 weeks) with AngII were used. As a negative control, we used rats that were never exposed to AngII (Figure 1A). In the second set of experiments, 2 control groups (Ctrl and Ctrl+Fasudil), and 2 experimental groups (AngII administered for 6 weeks and AngII+Fasudil administered for the last 4 weeks) were studied. Fasudil (100 mg/kg/day) was given in the drinking water (Figure 1B).

4.4. Blood Pressure Measurements

SBP was determined once a week, in the morning, in conscious pre-warmed restrained rats by non-invasive plethysmography (NIBP machine, IITC Inc., Woodland Hills, CA, USA) by the tail-cuff method. At least four determinations were made in every session and the mean of the four measurements was taken as the SBP value.

4.5. Renal Function Measurements

Plasma and urinary creatinine levels were measured by the Jaffé alkaline picrate assay (VALTEK Diagnostica, Santiago, Chile). Urinary protein concentration was determined by Bradford’s method (Bio-Rad protein assay, Kidlington, UK) [61]. Creatinine clearance was calculated according to the standard formula $C = \frac{U \times V}{P}$, where $C$ is the creatinine clearance, $U$ is the creatinine urinary concentration, $V$ is the urine flow rate per minute, and $P$ is the creatinine plasmatic concentration [58]. In the samples of urine and plasma, the amounts of Na$^+$ and K$^+$ were measured using the electrolyte meter 9180 Electrolyte Analyzer (Roche). To determine the fractional excretion of Na$^+$ and K$^+$, the following equation was applied:
FE Na⁺ or K⁺ = \frac{(PC \times [Na⁺]U or [K⁺]U)}{(CU \times [Na⁺]P or [K⁺]P)} \times 100

Where, [Na⁺]U or [K⁺]U = Concentration of Na⁺ or K⁺ in urine; and [Na⁺]U or [K⁺]U = Concentration of Na⁺ or K⁺ in plasma. PC = Plasma creatinine and CU = Creatinine in urine.

To determine the ratio U prot/U Crea, the following formula was applied:

\[ \frac{U_{Prot}}{U_{Crea}} = \frac{PU}{CU} \]

Where, PU = Protein in urine and CU = Creatinine in urine.

4.6. Thiobarbituric Acid Reactive Substances (TBARS) Measurement

TBARS were quantified by using a modified version of the method published by Ramanathan and collaborators. Plasma and supernatant of renal homogenates were mixed with acetic acid (20% v/v), SDS (8% w/v), and thiobarbituric acid (0.8% TBA w/v) and heated at 90°C for 60 min. Afterwards, the mixture was centrifuged to remove precipitated material, and absorbance was measured at 532 nm. TBARS were estimated with a calibration curve of MDA used as standard.

4.7. Histological Damage Assessment

Tissue damage was evaluated by a semi-quantitative morphometric analysis that was performed according to the immunoreactivity intensity observed in the kidney tissue sample for each of the antibodies used (OPN, ED-1, α-SMA, Col III). This was done from a score on a scale from negative to positive three (− to +++), defined according to the immunoreactivity intensity observed in the kidney tissue sample ([−] = 0–10%, [+] = 10–40%, [++] = 40–70% y [++++] = 70–100% of the area observed) [62–65]. To assess the degree of fibrosis, staining of collagen fibrils by Sirius red F3BA was carried out as previously described [66].

4.8. Tissue Processing and Immunohistochemical Analysis

Renal slices that included the cortex, medulla, and papilla, were fixed for 24 h by immersion in Bouin’s solution at room temperature. The tissue was then dehydrated, embedded in Paraplast plus (Monoject Scientific, St. Louis, MO, USA), serially sectioned at 5-mm thickness with a rotatory microtome, mounted on glass slides, and stored until immunostaining. Immunolocalization studies were performed using an indirect immunoperoxidase technique [67]. Tissue sections were quickly dewaxed, rehydrated, rinsed in immune solution (IS) (in M: 0.11 Na₂HPO₄, 0.04 KH₂PO₄, 1 NaCl, 0.32 Tris-HCl, and 0.03 sodium azide) pH 7.6, and incubated overnight at 22°C with the primary antibody (1:100). Afterward, sections were washed three times with IS for 5 min each, followed by 30 min incubation at 22°C with the corresponding secondary antibody (1:20) and with the peroxidase-anti-peroxidase (PAP) complex (1:150). Immunoreactive sites were revealed using 0.1% (w/v) 3.3′-diaminobenzidine and 0.03% (v/v) hydrogen peroxide solution. The antisera and PAP complex were diluted in IS containing 0.25% (v/v) Triton X-100 and 0.7% (w/v) λ-carrageenan. The sections were rinsed with IS buffer between incubations, counterstained with hematoxylin, dehydrated and cleared with xylene, and coverslipped. Images acquired using a Nikon Eclipse E600 microscope and Nikon DXM1200 digital camera were analyzed using conventional light microscopy and Nomarski differential interference contrast (DIC) microscopy, as previously described [68]. The Col III-stained area in each image was quantified utilizing computer-assisted image-analysis software (Simple PCI, Hamamatsu, Japan). Total immunostained cells were averaged and expressed as the mean absolute values per square micron, as previously described [68].

4.9. Enzyme-linked Immunosorbent Assay (ELISA)

ELISA assays were performed to determine the amount of IL-1β in the kidney. Rats were anesthetized with ketamine/xylazine (25:2.5 mg/kg of body weight, i.p.). Afterwards the kidney was removed and homogenized with an Ultra-Turrax homogenizer in buffer (ratio: 0.1 g renal tissue: 1 ml lysis buffer)
containing 100 mM Tris-HCl pH 7.4, 5 mM EDTA, 1% SDS, 1 μM PMSF and a protease inhibitor cocktail (Halt™ Protease Inhibitor Cocktail, Pierce, Rockford, IL, USA). Then, samples were centrifuged at 14,000 xg for 10 min (Eppendorf 5415C, Eppendorf, Hamburg, Germany). Supernatants were collected and protein content assayed by Bicinchoninic Acid (BCA) Protein Assay. Cytokine levels were determined by sandwich ELISA, according to the manufacturer’s protocol (IL-1β EIA kit, Enzo Life Science, USA). For the assay, 100 µL of samples were added per well and incubated at 4°C overnight. A calibration curve with recombinant cytokine was included. Samples were incubated with secondary antibody at room temperature for 2 h and the reaction was developed with avidin–HRP and substrate solution. Absorbance was measured at 450 nm with reference to 570 nm with the microplate reader Synergy HT (Biotek Instruments). The results were normalized by protein amount in ng/ml.

4.10. Western Blot Assays

Proteins of homogenized renal tissue samples (50 µg of proteins) were resolved by electrophoresis in 10% SDS-polyacrylamide gel, using one lane for the pre-stained molecular weight markers. Proteins were transferred to a PVDF membrane (pore size: 0.45 μm), blocked at room temperature with Tris pH 7.4, 5% skim milk (w/v) and 1% BSA (w/v). Then, the PVDF membrane was incubated overnight at 4°C with anti-Cx43 (1:1,000), anti-p-MYPT1 (1:500), or anti-MYPT1 (1:1,000) antibody, followed by incubation with secondary antibody conjugated to peroxidase (1:2,000) for 1 h at room temperature. Then, the PVDF membrane was stripped and reblotted with the anti-α-tubulin antibody (1:5,000) used as loading control, following the same procedure described above. After repeated rinses, immunoreactive proteins were detected with ECL reagents (Pierce Biotechnology, Rockford, IL, USA) according to the manufacturer’s instructions. The bands detected were digitized and subjected to densitometry analysis using the software Image J (Version 1.50i, NIH, Washington, DC, USA).

4.11. Statistical Analysis

For each data group, results were expressed as mean ± standard error (SE); n refers to the number of independent experiments. Detailed statistical results were included in the figure legends. Statistical analyses were performed using GraphPad Prism (version 7, GraphPad Software, La Jolla, CA). In case of multiple comparisons, data were analyzed by one or two-way analysis of variance (ANOVA) followed, in case of significance, by a Tukey’s post-hoc test. A probability of P<0.05 was considered statistically significant. Results are expressed as the average of values from each independent experiment ± SE.

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Author Contributions: Gonzalo I. Gómez, Victoria Velarde and Juan C. Sáez conceived and designed the experiments; Gonzalo I. Gómez performed the experiments; Gonzalo I. Gómez and Juan C. Sáez analyzed the data; Juan C. Sáez and Victoria Velarde contributed reagents/materials/analysis tools; Gonzalo I. Gómez, Victoria Velarde and Juan C. Sáez wrote the paper.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>AngII</td>
<td>Angiotensin II</td>
</tr>
<tr>
<td>CKD</td>
<td>Chronic kidney disease</td>
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<tr>
<td>Col III</td>
<td>Collagen type III</td>
</tr>
<tr>
<td>Cx GJs</td>
<td>Connexin gap junctions</td>
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<tr>
<td>Cx HCs</td>
<td>Connexin hemichannels</td>
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<tr>
<td>Cx43</td>
<td>Connexin 43</td>
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ED-1  Infiltration of macrophages
GFR  Glomerular filtration rate
IL-1β  Interleukin-1β
MDA  Malondialdehyde
MYPT-1  Myosin phosphatase target subunit 1
NFκB  Nuclear factor kappa-light-chain-enhancer of activated B cells
OPN  Osteopontin
OS  Oxidative stress
RAS  Renin angiotensin system
Rho GTPase  Rho family of small GTPases
ROS  Reactive oxidative species
TBARS  Thiobarbituric reactive species
TGF-β  Transforming growth factor beta
TNF-α  Tumor necrosis factor-α
UProt/UCrea  Ratio urine protein/urine creatinine
α-SMA  Alpha-smooth muscle actine

References


