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ANGIOTENSIN II TYPE 2 RECEPTOR AGONIST, COMPOUND 21, PREVENTS TUBULAR EPITHELIAL CELL DAMAGE CAUSED BY RENAL ISCHEMIA.

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Abbreviations: GFR, glomerular filtration rate; AKI, acute renal failure; IR, ischemia-reperfusion; RAS, renin angiotensin system; AngII, angiotensin II; AT1R, angiotensin II type 1 receptor; AT2R, angiotensin II type 2 receptor; C21, compound 21; MDCK, Madin-Darby canine kidney; FE, fractional excretion; PAS, periodic acid–Schiff

Abstract

During ischemic acute kidney injury (AKI), loss of cytoskeletal integrity and disruption of intercellular junctions are rapid events in response to ATP depletion. Angiotensin II type 2 receptor (AT2R) is overexpressed in injury situations and its stimulation by angiotensin II (AngII) is related to beneficial renal effects. Its role on ischemic AKI has not been deeply studied. The aim of the present study was to investigate whether pretreatment with the AT2R agonist, C21, prevents ischemic renal epithelial cell injury. Studies in a model of 40 min of renal ischemia followed by 24 h of reperfusion (IR) in rats demonstrated that C21 pretreatment attenuated renal dysfunction and induced better preservation of tubular architecture. In addition, we studied the expression of Rho GTPases, RhoA and Cdc42, since they are key proteins in the regulation of the actin cytoskeleton and the stability of epithelial intercellular junctions. IR downregulated RhoA and Cdc42 abundance in rat kidneys. C21 pretreatment prevented RhoA reduction and increased Cdc42 abundance compared to controls. We also used an *in vitro* model of ATP depletion in MDCK cells grown on filter support. Using immunofluorescence we observed that in MDCK cells, C21 pretreatment prevented the ATP depletion-induced reduction of actin in brush border microvilli and in stress fibers. Moreover, C21 prevented membrane E-cadherin reduction, and RhoA and Cdc42 downregulation. The present study describes for the first time a renoprotective effect of the AT2R agonist, C21, against AKI, and provides evidence supporting that stimulation of AT2R triggers cytoprotective mechanisms against an ischemic event.

Keywords

Acute kidney injury; ischemia; angiotensin II type 2 receptor; compound 21; tubular epithelial cell

1. Introduction

Acute kidney injury (AKI) is characterized by a sudden reduction in glomerular filtration rate (GFR). The high morbidity, mortality and healthcare costs, make AKI a major global health problem. Despite support measures and renal replacement therapies, there are still no pharmacological interventions to reduce mortality, facilitate recovery or prevent complications [1]. Renal ischemia reperfusion (IR) injury is one of the major causes of AKI [1,2].

Ischemic damage involves hemodynamic alterations, inflammation and damage to endothelial and epithelial cells, and it is followed by repair mechanisms that, in the best scenario, restore epithelial integrity. If those repair mechanisms are incomplete, the acute injury can lead to the development of chronic kidney damage [2].

Actin cytoskeleton is essential for the establishment and maintenance of cellular structure and function [3]. ATP depletion as a result of ischemia promotes the progressive disassembly of actin filaments through the activation and relocalization to the apical region of the actin depolymerizing factor (ADF)/cofilin [4]. A redistribution of the filaments occurs from the apical pole towards the cytoplasm, generating a progressive loss of the brush border, which could then detach from the apical membrane towards the tubular lumen [3]. Disruption of tight and adherens junctions and focal adhesions also occurs, both in response to loss of actin cytoskeleton integrity and to inflammatory mediators. Adherens junctions are important for the establishment of cell-cell junctions and apicobasal cell polarity [5]. Loss of this cell-cell junctions integrity contributes to backleak of glomerular filtrate and leads to tubular cells detachment from the basal membrane and tubular obstruction [2]. Tubular obstruction and backleak are, in part, responsible for reduced GFR [6].

Rho GTPases are a group of small GTPases implicated in the regulation of the actin cytoskeleton and the stability of epithelial intercellular junctions, which plays an important role in renal response to ischemia. This group of GTPases comprise more than 20 proteins including

Cdc42 and RhoA. By modulating the actin cytoskeleton, Rho GTPases participate in controlling cell shape, polarity, motility, and adhesion, among other functions [7]. ATP depletion has been demonstrated to inactivate these GTPases [8] and thereby could contribute to increased paracellular backleak of filtrate and tubular obstruction. RhoA is involved in stress fibers and focal adhesions formation and plays a key role in the establishment and maintenance of adherens and tight junction [8-10]. Cdc42 participates in actin reorganization and plays a key role in the establishment of both epithelial and migratory polarity [5,10]. Cdc42 is also involved in regulating membrane protein trafficking and in the formation and stabilization of intercellular junctions [10].

The renin-angiotensin system (RAS) regulates various organs functions, both systemically (regulation of blood pressure and electrolyte homeostasis) and through its local effects on tissues. Intrarenal RAS plays a key role in kidney physiopathology. Angiotensin II (AngII) is the main mediator of RAS and mediates its effects mainly by binding to two receptors: AngII type 1 receptor (AT1R) and AngII type 2 receptor (AT2R). Activation of these receptors stimulates different signaling pathways which produce different biological responses. AT1R mediates vasoconstriction and non-hemodynamic events such as inflammation, cell proliferation, apoptosis and fibrosis, which are crucial in kidney damage development, but are also necessary for the initiation of repair [11,12]. Several studies have described an increase in intrarenal AngII levels after renal IR damage [13,14]. In our laboratory we demonstrated RAS activation after renal IR and we reported that pretreatment with the AT1R antagonist, losartan, elicited protective effects against tissue damage and dysfunction. Losartan renoprotection was mainly mediated by its anti-inflammatory and anti-fibrotic effects [15,16]. Recently, similar treatment has been described to reduce the incidence of chronic kidney disease after ischemic AKI [17].

AT2R is widely expressed in the fetus where it is critical for organ development and differentiation. In adults, its expression decreases. However, in cortical, distal and collecting tubules its expression remains relatively abundant [18,19]. AT2R is overexpressed in injury situations and its stimulation is related to beneficial effects on the kidney [20]. Most AngII effects mediated by AT2R counteract the pathogenic effects mediated by AT1R. AT2R is involved in natriuresis and vasodilation mediated by the release of nitric oxide (NO) and bradykinin, has anti-inflammatory and antifibrotic actions, and participates in tissue repair [21]. Regarding ischemic AKI, the role of AT2R has not been deeply explored. AT2R overexpression was reported after renal IR [14]. To our knowledge the only study that investigated the role of AT2R in renal IR was performed in rats and described that the administration of the AT2R antagonist, PD123319, intensified renal blood flow reduction in rats [22].

The development of the non-peptide AT2R agonist, compound 21 (C21), has facilitated the study of this receptor. Moreover, this AT2R agonist is a potential therapeutic agent since it can be administered orally and it is highly specific [20,23]. C21 has demonstrated potent renal anti-inflammatory, antifibrotic, and antiapoptotic actions in animal models of various diseases, such as obesity [24], diabetic nephropathy [23], cyclosporine nephrotoxicity [25] and prevented renal inflammation in a model of endotoxemic AKI [26]. Furthermore, reported data demonstrated that C21 promoted beneficial effects after ischemic cerebral [27] and cardiac [28] damage.

Based on the above, our hypothesis assumes that the response of renal epithelial to ischemic damage is conditioned by the balance of AngII actions on AT1R and AT2R, and that acting via AT2R contributes to attenuate renal damage. Therefore, the aim of the present study was to investigate whether pretreatment with the AT2R agonist, C21, prevents ischemic renal epithelial cell injury. We used an *in vivo* rat model of IR to evaluate kidney function, epithelial structure alterations and Rho GTPases expression, and an *in vitro* model of ATP depletion in MDCK cells to study actin cytoskeleton integrity.

2. Materials and Methods

2.1. Animals and treatments

Experiments with rats were conducted according to protocols based on the Guide for the Care and Use of Laboratory Animals (8th Edition, Washington, DC: The National Academies, National Research Council, 2011) and approved by our Institutional Animal Care and Use Committee (Res. Nº 538/2018). Adult male Wistar rats (300-350 g) were maintained in standard conditions and were provided a standard diet and water *ad libitum*.

Rats were randomly distributed into the following groups (n=6 per experimental group): sham-operated control group (C), rats that underwent 40 min of unilateral renal ischemia followed by 24 h of reperfusion (IR), and rats treated during 2 days before ischemia with C21 (IR+C21) 0.3 mg/kg body weight (bw) per day or 1 mg/kg/day. C and IR group were treated with vehicle (saline). C21 (Vicore Pharma, Mölndal, Sweden) was dissolved in sterile saline solution to administer intraperitoneally (i.p.) the same volume. The last dose was administered 1 h before the ischemic insult. Doses used were chosen based on the demonstration of renoprotective effects in multiple experimental studies [23-25] without changes in mean arterial pressure [29].

Rats were anesthetized with ketamine (Kensol, Argentina)/xylazine (Alfasan, Woerden-Holland) 100/3 mg/kg bw i.p. Body temperature was kept constant (37 °C) by placing the animals on a heating pad. Right renal artery was clamped with a non-traumatic vascular clamp for 40 min [15,16]. Total ischemia was corroborated by observing the blanching and posterior darkening of the entire kidney surface. After 40 min, blood reflow was allowed by removing the clamp. Kidneys were observed to verify color change and thereafter abdominal incision was sutured. Once recovered from anesthesia animals were placed in individual cages with food and water *ad-libitum*. During the last 16 h of the 24 h reperfusion period, rats were housed in

metabolic cages (Nalgene Labware, Rochester, New York, USA) for urine collection. At the end of the reperfusion period, the rats were anesthetized with ketamine/xylazine 100/3 mg/kg bw, i.p. An abdominal incision was made, and blood was drawn from the inferior cava vein and the postischemic kidney was excised.

2.2. Renal function studies

Blood was immediately centrifuged to separate plasma for creatinine, Na^+, and K^+ measurements. Urine volume was measured gravimetrically. Urinary creatinine, Na^+, K^+, and protein were measured. GFR was estimated by creatinine clearance and was referred to 100 g bw. Creatinine measure was determined using a commercial kit (Creatinina, Wiener Lab, Rosario, Argentina). Na^+ and K^+ measurements were performed by an electrolyte analyzer with ion-selective electrodes (Roche Diagnostics, Switzerland). Urinary protein concentration was determined by a commercial kit based on pyrogallol red-molybdate (Proti U/LCR, Wiener Lab, Rosario, Argentina).

Fractional excretion of sodium (FE_{Na}), and potassium (FE_K) were calculated by relation between sodium and potassium clearance and GFR. Fractional excretion of water (FE_{H2O}) was calculated by relation between plasmatic and urinary creatinine. Urine protein excretion load ($E_{Protein}$) was also calculated.

2.3. Renal Histology

For histological preparations, a central portion of explanted kidneys were fixed in a 10% formaldehyde solution. The kidney sections were embedded in paraffin, sectioned at 3-5 μm thickness, and stained with periodic acid–Schiff (PAS) to study renal parenchyma by light microscopy. Tubular injury score was evaluated. The variables assessed were: tubular necrosis and dilation, loss of brush border and intratubular casts. The injury score was from 0 to 4: Score 0: normal; score 1: mild (<25%); 2: moderate (25-50%); 3: severe (50-75%) and 4: extensive damage (>75%).

2.4. Preparation of renal homogenates

After kidney removal, it was decapsulated and placed on a buffered sucrose medium (0.27 M sucrose, 5 mM Tris-HCl, 1 mM EDTA, 0.1 mM PMSF (Sigma Aldrich), 0.1 mM Leupeptin (Sigma Aldrich), pH 7.4). All subsequent steps were performed at 4 °C. Renal tissue was homogenized in buffered sucrose medium (5 ml g^{-1} of tissue) using a motor-driven Teflon-glass Potter homogenizer (10 strokes at 800 rpm) for immunoblotting analysis. Homogenates for RNA extraction were homogenized manually with a glass-Teflon homogenizer with 1 mL of Trizol (Life Technologies, GIBCO, USA). Aliquots of the homogenates were stored at −70ºC until use.

2.5. Immunoblotting analysis

Total protein concentration of the renal homogenates or MDCK cell lysates were measured with Coomasie brilliant blue G250 [30]. Immunoblotting was performed as previously described [31]. Briefly, 30 µg of protein were subjected to SDS 12% polyacrylamide gel electrophoresis. Gels were electrotransferred to polyvinyl difluoride (PVDF) membranes. To verify uniform protein loading and transference, membranes were stained with Ponceau 2R (Sigma Aldrich). Blots were blocked with 10% non-fat milk in PBS with 0.05% Tween. Then, they were probed with the primary mouse antibodies anti-Rho A (1:600, sc-418, Santa Cruz Biotechnology, USA), anti-Cdc42 (1:500, sc-8401, Santa Cruz Biotechnology, USA), anti-GAPDH (1:1000, sc-365062, Santa Cruz Biotechnology, USA) or anti-α tubulin (1:1000, sc-5286, Santa Cruz Biotechnology, USA). After washing, the blots were incubated with horseradish peroxidase-conjugated anti-mouse secondary antibody (1:2500, sc-516102, Santa Cruz Biotechnology, USA). Enhanced chemiluminescence (Amersham ECL Western Blotting Detection Reagent, GE Healthcare, USA) was used to detect protein bands. Autoradiographs were obtained by exposing the membranes to Kodak XAR films, and the bands were evaluated by densitometry using ImageJ program.

2.6. Real time PCR

Total RNA was isolated from renal homogenates using Trizol Reagent (Invitrogen, San Diego, USA) according to manufacturer's instructions. Reverse transcription of total RNA (1 μg) was performed with Moloney murine leukemia virus reverse transcriptase (M-MLV Easy Script RT, Transgen Biotech, China), conforming to the manufacturer's instruction. RT negative controls were performed by omitting the reverse transcriptase. Each cDNA sample was amplified in triplicate with a MiniOpticon real-time PCR system (BioRad, USA) using Syber Green Real Mix (Biodynamics, Argentina). Amplification profile: 2 min at 94°C, followed by 45 cycles of 94°C for 15 s, 60°C for 30 s, 72°C for 30 s, and a final extension at 72°C for 10 min. Primers sequences used were: AT2R sense 5'-CTGGCTGTGGCTGACTTACT-3', AT2R antisense 5'- CACTTTGCACATCACAGGTCC-3'; β-actin sense 5'-ATTGCTGACAGGATGCAGAA-3', β-actin antisense 5'-TAGAGCCACCAATCCACACAG-3'. PCR products length was checked on a 2% agarose gel.

Relative gene expression was calculated using the comparative ΔΔCt method. AT2R gene expression was normalized with respect to β-actin expression. No changes were found in the expression of β-actin RNA between the different experimental groups.

2.7. Cell culture and treatments

Madin-Darby canine kidney (MDCK) cells were obtained from Keith Mostov lab (UCSF, CA, USA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) with 4.5 g of glucose/l, supplemented with 5% fetal bovine serum (Gibco), 2 mM glutamine (Gibco), penicillin and streptomycin (Gibco). Cells were incubated at 37°C in a humidified atmosphere with 5% CO_2 and were passed twice a week. Cells were tested monthly by PCR for contamination with mycoplasma.

Cell hypoxia to simulate ischemia was achieved by chemical ATP depletion [32,33]. To achieve ATP depletion in cultured cells, inhibition of oxidative and glycolytic energy

metabolism is required. To reduce ATP levels, confluent cells were incubated in hypoxia medium containing DMEM, glutamine and antibiotics, without fetal bovine serum, supplemented with mitochondria inhibitor antimycin A (10 μM, Sigma Aldrich) and the glycolytic inhibitor 2-deoxyglucose (10 mM, Sigma Aldrich) by 90 min at 37 °C. As previously reported this treatment in MDCK cells reduces ATP stores to less than 10% of control [32,33].

Cells were divided into four groups (n=3 per experimental group): control group (C), control group with administration of 1 μM C21 (C21), ATP depleted group (I) and ATP depleted group with administration of 1 μM C21 (I+C21). C21 was dissolved in saline and added to the culture medium 24 h prior to ATP depletion. C21 dose was chosen based on previous studies [26,34].

2.8. Fluorescence microscopy

MDCK cells were grown on transwells containing 0.4 μm pore size polycarbonate membrane inserts (Corning Inc., USA) for 4 days. Cells were treated as described above. After treatment, cells on transwells were washed with PBS, fixed with 4% paraformaldehyde, permeabilized and blocked with 0.3% Triton X-100/1% bovine serum albuminin/PBS, pH 7.4, for 10 min. Then, they were incubated with mouse monoclonal antibodies against E-Cadherin (1:100 BD Biosciences, USA), for 2 h. The transwells were washed, incubated during 1 h with the secondary antibodies conjugated to Alexa 488 or phalloidin-Alexa 568 (1:200, Molecular probes-A34055, Thermo Fisher Scientific, USA) for actin staining and with 4′,6-diamidino-2-phenylindole (DAPI, 1:100, Invitrogen, Thermo Fisher Scientific, USA) for nuclear staining and mounted with ProLong (Invitrogen, Thermo Fisher Scientific, USA). Confocal laser microscopy (Nikon C1SiR with inverted microscope Nikon TE200) was used to observe fluorescence. 0.3-μm thick serial optical sections were collected in the z-axis. Z-stacks were built. Projections were obtained using ImageJ software. Brightness and contrast were equally

adjusted to the entire images using Adobe photoshop software to improve visualization of fluorescence in figures.

In polarized MDCK cells, we defined that the top 2 μm of the cells corresponds to the brush border. To analyze actin organization at the brush border, phalloidin staining present at the top 2 μm of the cells in the z-sections of the corresponding channel was quantified and related to total cell fluorescence [35]. In order to evaluate the actin stress fibers associated to the focal adhesions, we quantified the phalloidin staining present at three bottom z-sections of the cells, and related it to total cell fluorescence. Plasma membrane associated E-cadherin was quantified at the central 2 μm of the cell in the z-sections. Plasma membrane staining was relativized to total E-cadherin in these sections. At least 300 randomly selected cells distributed in 8 different fields per experiment were analyzed.

2.9. Statistical analysis

Results were expressed as means ± SEM. Statistical comparisons between groups were performed using one-way analysis of variance and Newman Keuls *post hoc* test. P-value ≤ 0.05 was considered statistically significant.

3. Results

3.1. Effects of IR and C21 pretreatment on AT2R mRNA expression

In an *in vivo* rat model of 40 min of unilateral renal ischemia followed by 24 h of reperfusion (IR), we evaluated AT2R mRNA expression and the effects of AT2R activation with C21 on its own expression. Fig. 1 shows mRNA expression of AT2R in renal tissue evaluated by Real Time PCR. We found that AT2R mRNA was upregulated by IR, suggesting that this receptor could have an important role in the physiopathology of renal IR. Moreover, the stimulation of AT2R with C21 before IR further increased its expression indicating a positive feedback of this receptor. These data pointed to C21 as a possible therapeutic agent.

3.2. C21 pretreatment protects against renal dysfunction induced by IR

To prove whether C21 has a renoprotective effect, we next evaluated renal function in rats submitted to IR. Fig. 2 shows the effects of C21 pretreatment on renal function parameters after 40 min of ischemia followed by 24 h of reperfusion. Based on other studies in different experimental models [23-25] we tested two doses of C21: 0.3 and 1 mg/kg/day.

Fractional excretion of water (Fig. 2A), sodium (Fig. 2B) and potassium (Fig. 2C), and urine protein excretion load (Fig. 2D) in IR kidneys were significantly higher than in controls. We found a C21 dose dependent improvement in these tubular parameters. These parameters returned to control values with the higher C21 dose assayed. GFR was markedly reduced after IR (Fig. 2E). The lower dose did not significantly improve GFR. However, pretreatment with C21 1 mg/kg/day promoted a significant improvement in GFR. No statistically significant difference was found in body weight values (Fig. 2F).

3.3. C21 pretreatment protects against renal tubular epithelium damage induced by IR

Renal histopathology was evaluated by light microscopy. Fig. 3 shows sections corresponding to cortical areas stained with PAS. Fig. 3A shows sections of a sham operated rat. After 1 day of reperfusion, the histological changes found at the level of the cortical tubules were severe. Micrographs revealed severe tubular damage, evidenced by large areas of brush border loss, vacuolization of tubular cell cytoplasm, cellular desquamation into the lumen, abundant intratubular casts and necrosis (Fig. 3B).

Pretreatment with C21 induces a better preservation of the tubular architecture evidenced by less necrosis and larger areas with brush border preservation (Fig. 3C and 3D), although some areas with necrosis and intratubular casts were still observed in postischemic

kidneys. Tubular injury of moderate intensity was observed with the lower dose while with the high-dose, injury was of mild to moderate intensity (Fig. 3E).

3.4. Effects of IR damage and C21 pretreatment on Cdc42 and RhoA protein abundance

As RhoA and Cdc42 are key proteins in the maintenance of the epithelial cell structure, we evaluated their abundance in renal tissue by Immunoblotting (Fig. 4). Since GAPDH was not significantly modified in any of the studied groups it was used as loading control. Cdc42 (Fig. 4A and 4B) and RhoA (Fig. 4A and 4C) protein expression was significantly reduced in postischemic kidneys. C21 pretreatment markedly prevented this reduction. Moreover, C21 was found to increase Cdc42 expression level compared to controls in a dose-dependent manner.

3.5. C21 pretreatment prevents renal epithelial cell damage induced by ATP depletion

In order to focus the study of the effects of C21 on the organization of the actin cytoskeleton and the stability of epithelial intercellular junctions, we utilized MDCK cell cultures. MDCK cells constitute a model of kidney tubule epithelial cells that has been well characterized and has been widely used to study actin cytoskeleton [32,33]. To simulate ischemia *in vitro*, polarized filter-grown MDCK cells were exposed to ATP depletion by incubating them with the mitochondrial inhibitor, antimycin A and the glycolytic inhibitor, 2-deoxyglucose. We analyzed actin and E-cadherin stainings by confocal microscopy (Fig. 5) in control and ATP depleted cells, and in cells pretreated with C21 before ATP depletion.

At the apical membrane, actin cytoskeleton organizes as parallel bundles of filaments, which constitutes tracks for myosin dependent transport of apical proteins [36] and provides the force to generate the membrane protrusion that constitutes the brush border [37]. The actin cytoskeleton associated with the brush border (Fig. 5A) was significantly diminished after ATP depletion and this decrease was prevented with C21 pretreatment (Fig. 5B). At the basal pole,

actin organizes as the stress fibers that stabilize interactions with the extracellular matrix [32]. Actin localized at the basal sections (Fig. 5A) was also decreased after ATP depletion and these alterations were prevented with C21 (Fig. 5B). E-cadherin is a key cell-cell adhesion molecule that undergoes rapid internalization in response to adherens junction disruption, reducing its membrane localization [38]. E-cadherin staining found at the basolateral membrane is shown in the center (Fig.5A). ATP depletion decreased E-cadherin localization at the basolateral membrane, and C21 prevented this decrease (Fig. 5C). Protein abundance of RhoA (Fig. 5D) and Cdc42 (Fig. 5E) in MDCK cell lysates was also evaluated. ATP depletion downregulated both Rho GTPases and this effect was partially prevented by C21.

4. Discussion

We have previously shown that RAS activation has an important role in the development of ischemic AKI, mediating inflammatory and profibrotic actions through AT1R [15,16]. As in many physiopathological situations, AngII via AT2R promotes opposed actions [21], in the present study we investigated whether AT2R activation could protect the kidney against ischemia. Our major finding was that AT2R agonist, C21, preserved renal tubular epithelial cell integrity in different models of ischemic injury. We demonstrated for the first time that stimulation of AT2R with C21 prevents renal dysfunction and morphological alterations in an *in vivo* model of IR and preserves actin cytoskeleton organization in ATP-depleted renal epithelial cells *in vitro*. Moreover, we provided additional evidence that C21 prevents RhoA and Cdc42 protein downregulation induced by ischemic damage, suggesting that Rho GTPases could be involved in C21 protective effect.

In the present study we showed that intrarenal AT2R mRNA is upregulated in response to an IR insult *in vivo*. AngII produced locally in the kidney could be responsible for this upregulation since a positive feedback mechanism for AT2R regulation has been described

[21]. AT2R overexpression might be part of a compensatory response of the kidney to ameliorate damage. Prior reports described that AT2R is overexpressed in various kidney injuries [39], including IR [14] and a role in tissue remodeling was demonstrated in other pathologies [20]. In addition, we showed that the administration of C21 previous to the IR insult further increased renal AT2R mRNA expression. It has been reported that NO increases AT2R transcription in vascular endothelial cells [40]. Since the activation of AT2R increases the synthesis and release of NO and cGMP, a positive feedback mechanism has been identified for the regulation of the expression of this receptor, so that its activation leads to an increase in its expression. Furthermore, in a model of hypertensive rats, the stimulation of AT2R, either with C21 or AngII, generated an increase in the renal expression of this receptor [41]. Our results are in agreement with these findings, providing additional evidence that, unlike other receptors, including AT1R, a positive feedback mechanism regulates AT2R to maintain and enhance its responses. We hypothesized that AT2R activation with C21 before IR damage would be protective and could be useful for minimizing short-and long-term damage.

In another series of experiments we tested whether C21 pretreatment could ameliorate kidney dysfunction and histopathological alterations induced by IR in rats. Our results showed that C21 pretreatment attenuated renal dysfunction. As regards to tubular function, we found that C21 pretreatment also attenuated renal epithelial damage after IR in a dose-dependent manner, as demonstrated by the improvement of tubular function parameters, which was associated with better preservation of the tubular architecture, less tubular necrosis and larger areas with preservation of the brush border. In fact, the 1 mg/kg dose, which is the highest dose used in other studies in rats that conserves AT2R specificity [24,29], completely prevented the IR induced increase in electrolyte and water fractional excretion and urine protein excretion load. Regarding glomerular filtration, we found that, although the highest C21 dose significantly improved the fall in GFR, this parameter did not reach control values, in

accordance with some intratubular casts and a certain degree of tubular obstruction that can still be observed at this reperfusion time.

The regulatory proteins Rho GTPases, Cdc42 and RhoA, play a key role in the maintenance of renal tubules epithelial integrity. Hallet et al. [42] demonstrated that Rho GTPases, including RhoA and Cdc42, get inactivated after an ischemic damage. It was also reported that RhoA and Cdc42 expression decreases after IR in rats [43]. Cdc42 is required to maintain epithelial polarity. In a model of IR, it was reported that Cdc42 was essential for forming adequate tubular structures since its absence generates hyperproliferative multilamellar structures, without recovery of renal function [44]. Moreover, Cdc42 is required for regulation of membrane trafficking and formation of intercellular junctions [10, 45]. Although still controversial, the role of RhoA in ischemic kidney injury has been widely investigated. It has been described that RhoA is inactivated by ischemia [42] and that the expression of a constitutively active form in renal proximal cells preserved cortical actin, stress fibers and tight junctions after ATP depletion [8,46]. RhoA activates its downstream effector Rho associated coiled-coil–forming protein kinase (ROCK) which initiates a signaling cascade that ends with ADF/cofilin inactivation [9]. After ATP depletion this signaling cascade is disrupted promoting cytoskeleton breakdown [42]. On the other hand, many studies demonstrated that AngII, via AT1R, also activates RhoA/ROCK pathway which has been implicated in the regulation of proliferation, migration, inflammation and apoptosis in renal cells and that ROCK inhibitors directed to renal tubular cells have been shown to attenuate IR damage [47].

Taking into consideration the involvement of Cdc42 and RhoA in the renal epithelium response to ischemic injury, we evaluated the effects of C21 on the expression of these Rho GTPases in our experimental models. Consistent with a study that reported that the abundance of RhoA and Cdc42 proteins decreased after ischemia followed by one hour of reperfusion [43], in the present study we found a decrease in the relative abundance of the Cdc42 and RhoA in

postischemic kidneys after one day of reperfusion. Remarkably, C21 prevented the decrease in these Rho GTPases abundance. The reduction in RhoA and Cdc42 could be, at least in part, responsible for tubular alterations evidenced by histological studies. Cdc42 reduction after IR could be involved in E-cadherin loss from plasma membrane since it participates in the endocytosis and recycling of this protein, conditioning the stability of adherens junctions [48,49].

The modulation of signaling pathways that involve Rho GTPases would be important in renoprotection against ischemic injury. Whether these proteins participate in AT2R signaling in renal cells has not yet been elucidated. There is evidence demonstrating that AngII via AT2R activates focal adhesion kinase (FAK), inducing focal contacts formation and cytoskeleton organization mediated by RhoA and Cdc42, which together increase mesenchymal stem cells migration [50]. Our data indicate that C21, through activation of AT2R, promotes the enhanced expression of Cdc42, necessary for the regeneration of kidney tissue.

Consistently with our *in vivo* results, Cdc42 and RhoA protein abundance was downregulated in ATP depleted MDCK cells, and C21 pretreatment prevented this decrease in Rho GTPases expression. Consequently, we studied the effects of this AT2R agonist on the organization of the actin cytoskeleton and the stability of adherens junctions in MDCK cells grown on filter support. This is a well characterized model of polarized renal tubular epithelial cells and extensively used as an *in vitro* model of renal ischemia by chemical ATP depletion [32,33,38]. As previously described [4,8,32,51], we found that ATP depletion promoted actin cytoskeleton disruption. Stress fibers are important elements of the basal actin network in renal epithelial cells, which is involved in cell-matrix adhesion. Alterations in actin stress fibers are responsible for cell detachment that occur after ischemia [51]. After 90 min of ATP depletion we observed a marked reduction in actin stress fibers. In addition, we found that apical actin, which constitutes the core of microvilli structure, was reduced. The loss of brush border is a

characteristic change after ATP depletion in this model [32]. We also observed that ATP depleted cells presented decreased E-cadherin association to the basolateral membrane, suggesting disruption of adherens junctions. Other studies reported the internalization of E-cadherin in MDCK cells in response to ATP depletion [38]. The decreased Cdc42 and RhoA protein abundance observed in our *in vitro* model could be, at least in part, responsible for actin cytoskeleton and cellular junctions alteration induced by ischemia. Remarkably, C21 pretreatment prevented the actin cytoskeleton disruption at the brush border and the loss of stress fibers at the basal pole, as well as the reduction of E-cadherin localization at the basolateral membrane, and Cdc42 and RhoA downregulation induced by ATP depletion in MDCK cultures. Taken together, these results suggest a direct role of AT2R on actin cytoskeleton organization and maintenance of adherens junctions in tubular epithelial cells, by mechanisms involving Rho GTPases expression.

Considering that inflammation plays a key role in ischemic AKI pathophysiology [1,2] and actin cytoskeleton integrity [52], we could not disregard the anti-inflammatory effect of C21. These reported effects include reduced production of inflammatory cytokines and inflammatory cellular infiltrate in different experimental models [26,34,41]. Further research will be of great interest to elucidate the AT2R-mediated renoprotective mechanisms against ischemic injury.

4.1 Conclusion

The role of AT2R in the renal response to AKI remains poorly understood. The present study describes for the first time a renoprotective effect of the AT2R agonist, C21, against ischemic injury, and provides evidence supporting that stimulation of AT2R triggers cytoprotective mechanisms against an ischemic event. Presumably, actions that prevent the disruption of the actin cytoskeleton with the consequent loss of cell polarity, loss of cell-cell and cell-matrix contact, could protect against the structural and functional alterations observed

in postischemic kidneys. Our findings suggest a novel role of AT2R in the response to renal ischemic damage which has not been described so far.

CRediT authorship contribution statement

M. Fernanda Fussi: Investigation, Methodology, Data curation, Writing - original draft. **Florencia Hidalgo:** Investigation. **Gabriel Buono:** Investigation. **Susana B. Marquez:** Investigation. **Alejandro Pariani:** Investigation. **Jorge L. Molinas:** Conceptualization, Funding acquisition. **M. Cecilia Larocca**: Data curation, Conceptualization, Writing - review & editing, Funding acquisition, Supervision. **Liliana A. Monasterolo:** Investigation, Methodology, Conceptualization, Writing - review & editing, Funding acquisition, Supervision. **Sara M. Molinas:** Investigation, Methodology, Conceptualization, Writing - review & editing, Funding acquisition, Supervision.

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Conflict of Interest

The authors declare no conflicts of interest.

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Figure legends

Fig. 1. Effects of renal ischemia-reperfusion and C21 pretreatment on AT2R mRNA expression. Renal mRNA levels of AT2R in rats after sham operation (C), after 40 min of ischemia followed by 24 h reperfusion (IR) and in rats pretreated with C21 for 2 days previous to IR (IR+C21) were assessed by Real Time PCR as described in Materials and methods. AT2R mRNA levels were normalized to β-actin levels. Control values were assumed as 1. Data are expressed as mean ± SEM (n=6), $*$ $p<0.05$ compared with C and $^{\#}p<0.05$ vs IR.

Fig. 2. Effect of C21 pretreatment on renal dysfunction induced by ischemia-reperfusion. Rats were treated with C21 (0.3 or 1 mg/kg body weight, i.p.) or vehicle 2 days before induction of 40 min of renal ischemia followed by 24 h of reperfusion (IR+C21 or IR, respectively). Control group (C) was submitted to sham operation. (A-C) Fractional water, sodium, and potassium excretion (FEH_2O, FENa, FEK) respectively, (D) Urine protein excretion load ($E_{Protein}$), (E) Glomerular filtration rate (GFR) and (F) body weight. Results are expressed as mean ± SEM (n = 6). *$p<0.05$ vs C, $^{\#}p<0.05$ vs IR and $^{f}p<0.05$ vs IR+C21 0.3 mg/kg.

Fig. 3. Effects of C21 pretreatment on renal histological alterations induced by ischemia-reperfusion (IR). Histological study was performed by light microscopy in tissue sections stained with PAS from kidneys of sham operated rats (A) and kidneys submitted to 40 min of ischemia followed by 24 h of reperfusion (B). Histological characteristics in kidneys from C21 0.3 mg/kg/day and 1 mg/kg/day pretreated animals submitted to renal IR (C and D, respectively). Following IR, kidneys presented signs of acute tubular necrosis, i.e. tubular dilation, swelling and necrosis, loss of brush border and cell desquamation (arrow) in addition to intraluminal brush border debris and protein casts (*). Arrowhead shows preservation of brush border and tubular architecture in C and D. Scale bar represents 50 µm. E) Quantitation of tubular injury score (n=6). *$p<0.05$ vs C, $^{\#}p<0.05$ vs IR and $^{\int}p<0.05$ vs IR+C21 0.3 mg/kg.

Fig. 4. Effects of renal ischemia-reperfusion (IR) and C21 pretreatment on Cdc42 and RhoA protein abundance. A) Representative immunoblottings showing the expression of renal Cdc42 and RhoA in rats submitted to sham operation (C) or 40 min of renal ischemia followed by 24 h of reperfusion (IR) and in rats pretreated with C21 0.3 mg/kg/day and 1 mg/kg/day i.p. for 2 days before IR (IR+C21). GAPDH served as a loading control. Densitometric analysis for B) Cdc42 and C) RhoA. Ratio between RhoA or Cdc42 and GAPDH abundance was expressed as percentages of control group and represent means ± SEM (n=6), $*p<0.05$ vs C, $^{\#}p<0.05$ vs IR and $^{\int}p<0.05$ vs IR+C21 0.3 mg/kg.

Fig. 5. **Effect of C21 pretreatment on renal epithelial cell damage induced by ATP depletion**. A) Confocal microscopy images showing actin (red) and E-cadherin (green) stainings in polarized filter-grown MDCK cells. MDCK were pretreated with vehicle (I) or C21 (1 µM) for 24 h and then exposed to chemical ATP depletion for 90 min (I+C21). Control cells were treated with vehicle (C) or C21 (C21). Images shown in the upper, middle, and lower rows

were selected from the apical, central, and basal planes of the Z-stacks, respectively. Boxed areas in dashed line show views of a cell. Scale bar represents 10 μm. B) Bars represent F-actin fluorescence intensity at the top 2 μm of the cell (microvilliar actin), and fluorescence intensity of F-actin at the bottom of the cell, which corresponds to the stress fibers at the basal membrane area. In both cases, F-actin fluorescence was expressed as a percentage of total cellular F-actin. C) Bars represent E-cadherin in the basolateral membrane present in the central sections of the cell (central 2 μm) expressed as a percentage of total cellular E-cadherin in these sections. D) Representative immunoblottings and densitometric analysis for RhoA. Ratio between RhoA and α-tubulin abundance relativized to control abundance is shown for each group. E) Representative immunoblottings and densitometric analysis for Cdc42. Ratio between Cdc42 and α-tubulin abundance relativized to control abundance is shown for each group. Data are shown as mean ± SEM (n=3). *$p<0.05$.

CRediT authorship contribution statement

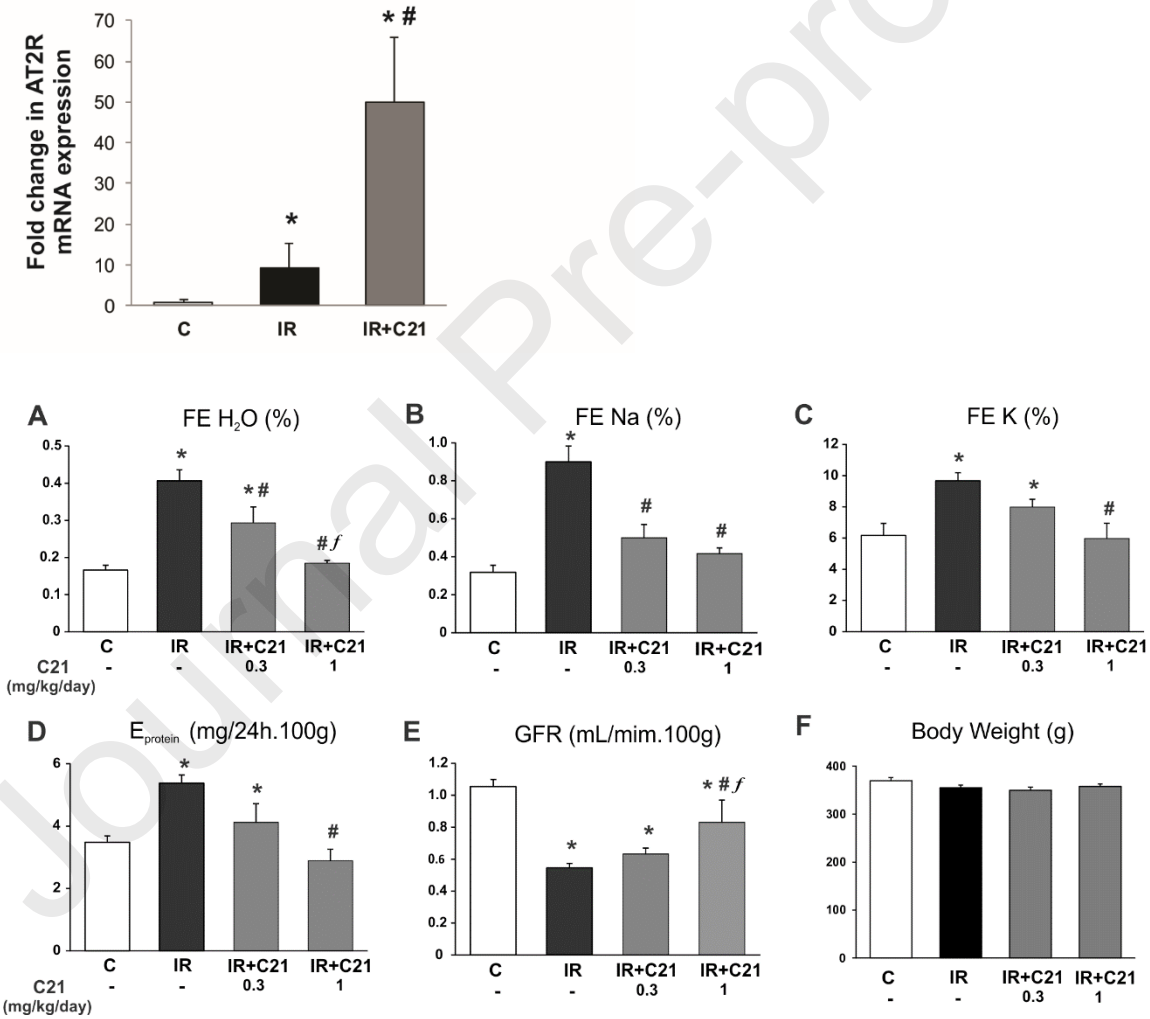
M. Fernanda Fussi: Investigation, Methodology, Data curation, Writing - original draft. **Florencia Hidalgo:** Investigation. **Gabriel Buono:** Investigation. **Susana B. Marquez:** Investigation. **Alejandro Pariani:** Investigation. **Jorge L. Molinas:** Conceptualization, Funding acquisition. **M. Cecilia Larocca**: Data curation, Conceptualization, Writing - review & editing, Funding acquisition, Supervision. **Liliana A. Monasterolo:** Investigation, Methodology, Conceptualization, Writing - review & editing, Funding acquisition, Supervision. **Sara M. Molinas:** Investigation, Methodology, Conceptualization, Writing - review & editing, Funding acquisition, Supervision.

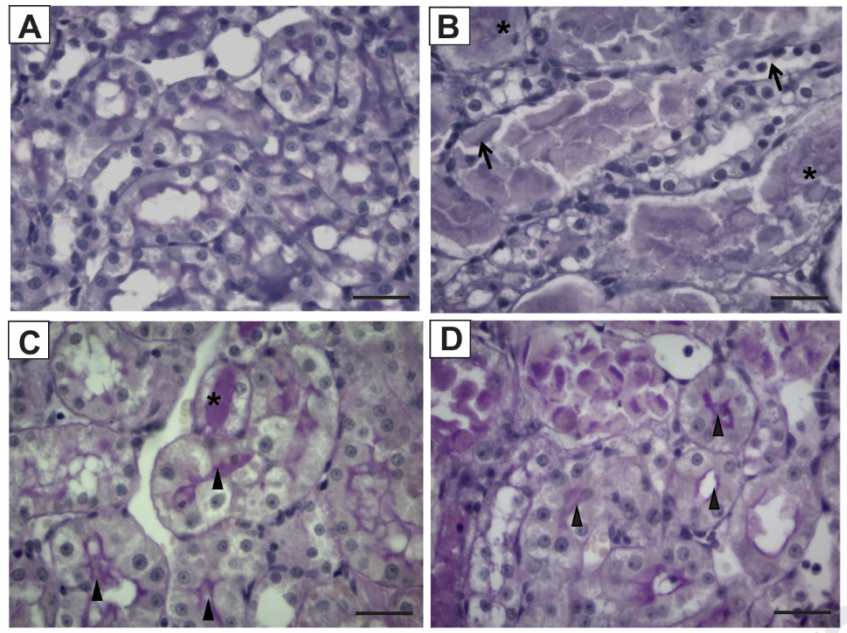
ANGIOTENSIN II TYPE 2 RECEPTOR AGONIST, COMPOUND 21, PREVENTS TUBULAR EPITHELIAL CELL DAMAGE CAUSED BY RENAL ISCHEMIA.

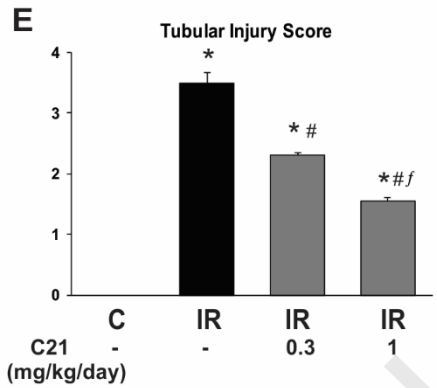
Fussi María Fernanda, Hidalgo Florencia, Buono Gabriel, Marquez Susana Beatriz, Pariani Alejandro, Molinas Jorge Luis, Larocca María Cecilia, Monasterolo Liliana Alicia, Molinas Sara María

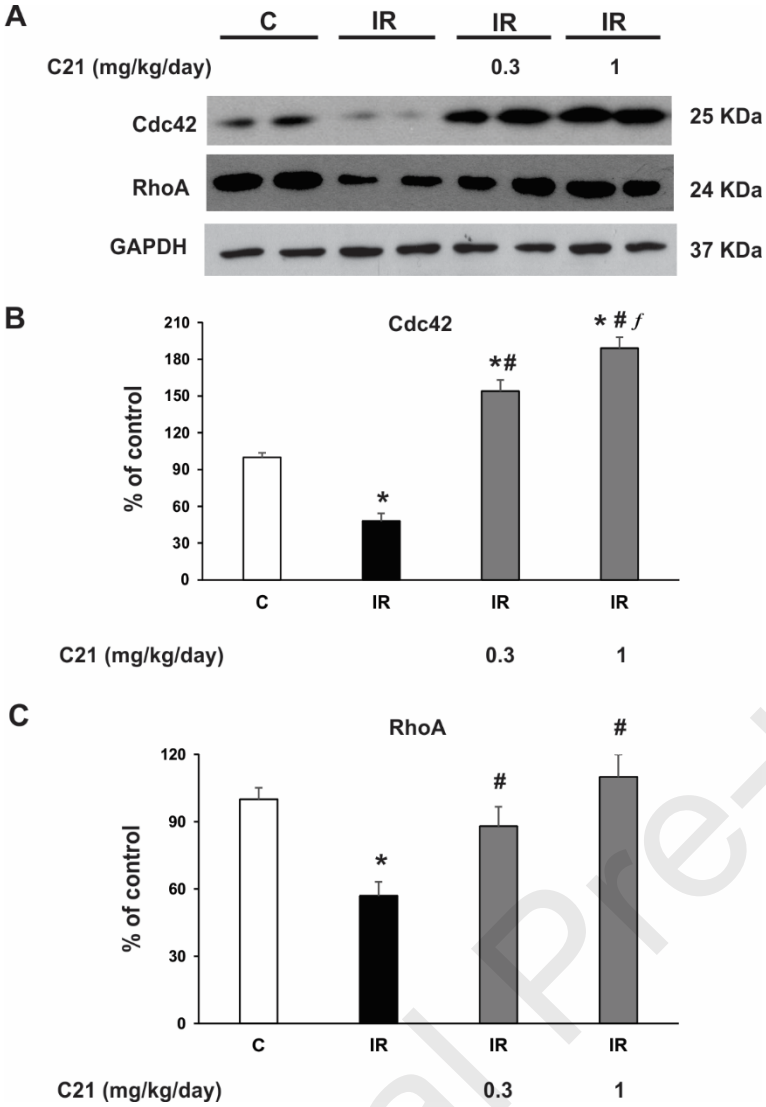
Conflict of Interest

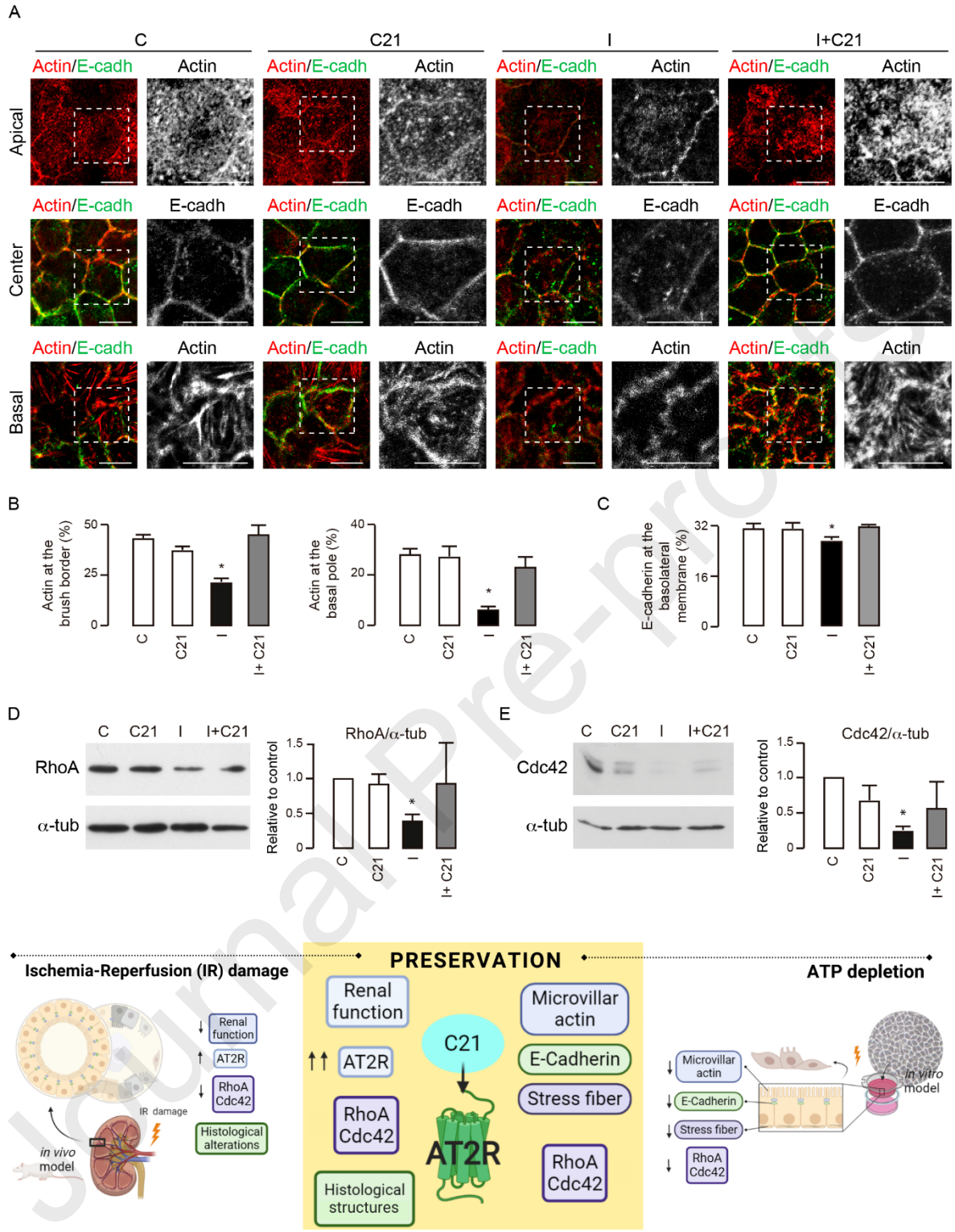
The authors declare no conflicts of interest.










A
C
C21
I
I+C21
Actin/E-cadh
Actin
E-cadh
Apical
Center
Basal
B
Actin at the brush border (%)
Actin at the basal pole (%)
C
C21
I
I+ C21
C
E-cadherin at the basolateral membrane (%)
D
RhoA
α-tub
RhoA/α-tub
Relative to control
E
Cdc42
Cdc42/α-tub
Ischemia-Reperfusion (IR) damage
PRESERVATION
ATP depletion
Renal function
AT2R
RhoA Cdc42
Histological alterations
IR damage
in vivo model
C21
Microvillar actin
E-Cadherin
Stress fiber
Histological structures
in vitro model