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Original Paper

1400W Prevents Renal Injury in the Renal Cortex But Not in the Medulla in a Murine Model of Ischemia and Reperfusion Injury

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Key Words
Ischemia/reperfusion injury • Inflammation • Macrophages polarization • 1400W

Abstract
Background/Aims: Acute kidney injury (AKI) carries high morbidity and mortality, and the inducible nitric oxide synthase (iNOS) is a potential molecular target to prevent kidney dysfunction. In previous work, we reported that the pharmacological inhibitions of iNOS before ischemia/reperfusion (I/R) attenuate the I/R-induced AKI in mice. Here, we study the iNOS inhibitor 1400W [N-(3-(Aminomethyl)benzyl] acetamide, which has been described to be much more specific to iNOS inhibition than other compounds. ***Methods:*** We used 30 minutes of bilateral renal ischemia, followed by 24 hours of reperfusion in Balb/c mice. 1400w (10 mg/kg i.p) was applied before I/R injury. We measured the expression of elements associated with kidney injury, inflammation, macrophage polarization, mesenchymal transition, and nephrogenic genes by qRT-PCR in the renal cortex and medulla. The Periodic Acid-Schiff (PAS) was used to study the kidney morphology. ***Results:*** Remarkably, we found that 1400W affects the renal cortex and medulla in different ways. Thus, in the renal cortex, 1400W prevented the I/R-upregulation of 1. NGAL, Clusterin, and signs of morphological damage; 2. IL-6 and TNF-α; 3. TGF-β; 4. M2(Arg1, Erg2, cMyc) and M1(CD38, Fpr2) macrophage polarization makers; and 5. Vimentin and FGF2 levels but not in the renal medulla. ***Conclusion:*** 1400W conferred protection in the kidney cortex compared to the kidney medulla. The present investigation provides relevant information to understand the opportunity to use 1400W as a therapeutic approach in AKI treatment.

Introduction

Acute kidney injury (AKI) is a group of syndromes defined by an abrupt decrease in glomerular filtration and is associated with considerable morbidity, mortality, and high

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costs [1]. Renal ischemia-reperfusion (I/R) injury is a significant cause of AKI, which is triggered by a transient reduction of blood flow followed by blood reperfusion. I/R injury can lead to acute cell death, tissue injury, and renal dysfunction [2, 3]. Renal I/R is involved in transitioning from AKI to chronic kidney disease (CKD) [4]. The clinical intervention addressed fluid management, but there are no specific therapies for each type of AKI [5]. Effective drugs to protect patients against the renal damage induced by ischemia and reperfusion are still lacking. Therefore, research is needed to find effective drugs to prevent and treat AKI.

The nitric oxide (NO) produced by iNOS provokes kidney tissue injury because NO combines with the superoxide radical and forms the cytotoxic metabolite, peroxynitrite, which causes cell membrane damage through protein nitration. Thus, the iNOS inhibition should ameliorate kidney damage [6-10]. Previously, we demonstrated that the pharmacological inhibition of iNOS with L-NIL (L-N(6)-(1-iminoethyl)lysine), a selective inhibitor of iNOS enzymatic activity, decreases the signs of renal damage induced by renal I/R in mice, reducing the oxidative stress and inflammatory pathway (TLR4 and IL-1β) [6]. Recently, we showed that aminoguanidine (AG), another iNOS inhibitor, protects the kidney injury induced by renal I/R in mice. AG recovered the GSH/GSSG ratio, the GST activity, and lipoperoxidation, preventing iNOS and Hsp27 upregulation. Moreover, AG inhibits the inflammation markers (IL-6, FOXP3, and IL-10 mRNA) upregulation [7]. In addition, other investigations have demonstrated that inhibiting the iNOS activity with specific iNOS inhibitors reduces oxidative stress, renal injury, and kidney dysfunction provoked by I/R or sepsis [8-10]. Moreover, iNOS knock-out animals are more resistant to kidney damage elicited by I/R than their wild-type counterparts [11]. Thus, the current information shows that iNOS inhibition prevents oxidative stress, inflammation, and kidney dysfunction observed by I/R. Therefore, it is necessary to increase the evidence to establish iNOS as a molecular target to prevent and treat AKI in humans. In this paper, we bet on the inhibitor 1400W, which has been reported to be low toxicity and is much more specific than other iNOS inhibitors [12-14]. Thus, in a model of cardiac I/R injury, 1400W (20 mg/kg) inhibited the NO production in mice treated with lipopolysaccharide (LPS) [15]. Besides, in a rat model of liver transplantation, the production of reactive nitrogen species (RNS), necrosis, and apoptosis was blocked by 1400W (5 μM) [16]. In addition, 1400W (20 mg/kg, rats) significantly reduced the volume of ischemic brain lesions, attenuated weight loss, and neurological dysfunction [17]. Besides,1400W (10 mg/kg) also reduced oxidative stress and the ischemia-reperfusion injury in an ex-vivo porcine donation model after the kidney donor's circulatory death [18]. Moreover, a comparative study using melatonin (a powerful antioxidant, iNOS inhibitor, and a scavenger of peroxynitrite) and 1400W observed that both melatonin and 1400W (10 mg/kg) were efficient in ameliorating experimental I/R injury, including oxidative and nitrosative stress in kidneys. Moreover, melatonin was more effective than 1400W, possibly through scavenging free oxygen radicals and peroxynitrite [19]. In the present study, we explore the protective effect of 1400W against I/R separately in the renal cortex and medulla.

During the I/R injury, the tubular epithelial cells experience acute tubular necrosis (ATN) and activate the regeneration process against cell damage [20]. Epithelial cells of the proximal convoluted tubule undergo a sequence of events, including cell dedifferentiation and proliferation, followed by cell migration, redifferentiation, and repairing the damaged epithelium [21, 22]. Interestingly, during the repair process, re-expression of proteins that participate during kidney embryonic development (nephrogenes) has been observed [23-25]. These proteins include, among others, mesenchymal factors, such as vimentin [6, 7], fascin-1, and FGF-2 [25, 26]. Moreover, we previously found that AG treatment before ischemia/reperfusion significantly prevented the vimentin and fascin-1 upregulation induced by I/R [7].

On another side, I/R triggers inflammation and leukocyte infiltration inside the tubulointerstitial compartment [27]. The proinflammatory macrophages (M1 phenotype) increase after 1 hour of reperfusion, peaking at 24 hours and persisting for 7 days in

mice kidneys. In contrast, M2 macrophages are present about 3-5 days after the initial injury, inducing cell proliferation and tissue repair in the kidney by secreting anti-inflammatory cytokines [28-30]. Interestingly, several immune cells express iNOS, among them macrophages, dendritic cells, and NK cells [31]. Although NO derived from iNOS is harmful in the kidney, an interesting work demonstrated that iNOS deficient mice exhibited enhanced M1 macrophage polarization without significant effects on M2 macrophages. Additionally, the iNOS inhibitor L-NIL significantly enhanced M1 macrophage polarization *in vitro*, suggesting that iNOS deficiency results in more severe inflammation [32]. Another study using a rat model of neuropathic pain showed that the treatment with 1400W (20 mg/kg) increased the plasma concentration of anti-inflammatory cytokines (IL-10) and pro-inflammatory cytokines (IL-1α, and IL-1β), suggesting that 1400W could alter the balance between pro- and anti-inflammatory cytokines [33]. Thus, the effect of 1400W in renal I/R is not clearly understood.

In this work, we investigated the role of 1400W in kidney injury, inflammation, macrophage polarization, mesenchymal transition, and nephrogenes separately in the renal cortex and medulla in a mice model of I/R. Remarkably, 1400W treatment reduced the I/R-activation signs of renal damage (tissue morphology, NGAL, and Clusterin expression), mesenchymal transition (vimentin and fascin-1), inflammation, macrophage polarization (M2/M1), and nephrogenes (FGF-2) in the renal cortex but not in the renal medulla. Therefore, the present investigation provides relevant information to propose to 1400W as an element for a therapeutic approach in AKI treatment.

Materials and Methods

Animals

Male Balb/c mice (20-25g and 2 months old) were housed in a 12 h light/dark cycle. Animals had food and water ad libitum and were maintained at the University de los Andes-Animal Care Facility [6, 7, 34]. All experimental 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. The National Institutes of Health). All procedures were approved by the Committee on the Ethics of Animal Experiments of the Universidad de los Andes, Chile.

Ischemia-reperfusion (I/R)

The animals were anesthetized with a volatile anesthetic (sevoflurane) and maintained on a 37°C blanket during the surgical procedure. A flank incision exposed both kidneys, and the renal pedicle was occluded for 30 minutes with a non-traumatic vascular clamp (cat N° 18055-02 Fine Science Tools). Renal blood flow was re-established (reperfusion phase) by clamp removal, and both incisions were sutured. Sham animals did not undergo renal pedicle occlusion [6, 7]. Mice were treated intraperitoneally (i.p.) with either vehicle (physiological saline) or 10mg/kg of 1400W [18, 19] (from MedChem Express, catalog HY-18731) 30 min before sham or 30 min of ischemia and 24 hours of reperfusion. Then, the animals were subjected to 24 hours of reperfusion.

Real-Time PCR

The experimental process was carried out according to how we describe before [35]. In brief, total RNA was isolated using a RNeasy Mini Kit (Cat Nº: 74104, Qiagen) according to the manufacturer's directions. Extracted RNA was quantified at 260 nm in a Spectrophotometer (NanoDrop One, Thermo Scientific), and the RNA's integrity (28S/18S ratio) was assessed by agarose gel electrophoresis. cDNA was prepared from total RNA (1,0 μg) using a Improm-IITM Reverse Transcription System (Cat Nº: A3800, Promega) and random hexamers primers. Then, PCR was performed duplicated for each experiment (Brilliant III Ultra-Fast SYBR® Green QPCR Master Mix (Cat Nº: 600882, Agilent). Amplicons were detected for Real-Time Fluorescence Detection (Rotor-Gene Q, Qiagen). The primers used are detailed in Table 1. Relative mRNA expression of the target genes was calculated using the $-2\Delta\Delta$ Ct method after normalization to the levels of 18S.

Morphological studies

Mice were anesthetized, as mentioned before, and kidneys were removed after tying the renal pedicle and then cut by a sagittal section in two halves, fixed in 10% formalin, included in paraffin, sectioned, dewaxed, rehydrated, and rinsed in water. After the pieces were dehydrated, they were embedded in paraffin, cut into 4-nm sections, mounted on glass slides, and stained with Hematoxylin and eosin (H/E) and Periodic Acid-Schiff (PAS) performed for light microscopy analysis. Morphological changes were analyzed blindly by a histologist (co-author).

Statistical analysis

Differences between groups were analyzed using the non-parametric Kruskal-Wallis ANOVA and posthoc Tukey test using GraphPad Prism Software. The level of significance was set at $p < 0.05$.

Table 1. List of primers used for RT-PCR

Gene	Sequence	Type	Tm (°C)	Species	Amplicon size (bp)
NGAL	5'-TGTCGCTACTGGATCAGAACA-3'	Forward	55.6	Mouse	85
	5'-GTACCTGAGGATACCTGTGCAT-3'	Reverse	56.1	Mouse	
Clusterin	5'-TTGACTCTGACCCCATCACA-3'	Forward	55.7	Mouse	110
	5'-GCTTTTCCTGCGGTATTCCT-3'	Reverse	55.4	Mouse	
IGFBP7	5'-TACAGTGGTTGATGCCCTCCAT-3'	Forward	58.3	Mouse	133
	5'-AGGCAAGAGCAGGGTTATAGCT-3'	Reverse	58.1	Mouse	
iNOS	5'-ACACTCTTCACCACAAGGCC-3'	Forward	57.6	Mouse	95
	5'-GGGTCCTCTGGTCAAACTCTTG-3'	Reverse	57.4	Mouse	
IL-1β	5´-TGCCACCTTTTGACAGTGATGAGA-3´	Forward	58.6	Mouse	102
	5´-CATCAGGACAGCCCAGGTCAAA-3´	Reverse	59.4	Mouse	
IL-6	5´-TACCATAGCTACCTGGAGTAC-3´	Forward	52.6	Mouse	150
	5´-AATTGGGGTAGGAAGGACTAT-3´	Reverse	52.7	Mouse	
TNF-α	5'-TGATCGGTCCCCAAAGGGATGA-3'	Forward	60.3	Mouse	101
	5'-CTGCTCCTCCACTTGGTGGTTT-3'	Reverse	59.6	Mouse	
FOXP3	5´-AACCTGAGCCTGCACAAGTGCTTT-3´	Forward	61.6	Mouse	134
	5´-TGAGGTCAAGGGCAGGGATTGGA-3´	Reverse	62.4	Mouse	
IL-10	5'-GCTCCAAGACCAAGGTGTCTAC-3'	Forward	57.4	Mouse	110
	5'-ACACACTGCAGGTGTTTTAGCT-3'	Reverse	57.1	Mouse	
TGF-β	5'-CGAAGCGGACTACTATGCTAA-3'	Forward	54.0	Mouse	130
	5'-TTCCCGAATGTCTGACGTATT-3'	Reverse	53.8	Mouse	
ARG-1	5'-AATGGAAGAGTCAGTGTGGTGCTG-3'	Forward	59.2	Mouse	116
	5'-TCAGTGTGAGCATCCACCCAAATG-3'	Reverse	59.5	Mouse	
EGR-2	5'-TTGACCAGATGAACGGAGTGGC-3'	Forward	59.5	Mouse	104
	5'-GATGGGAGCGAAGCTACTCGGATA-3'	Reverse	59.9	Mouse	
c-Myc	5'-TTTGGGGACAGTGTTCTCTGCCTC-3'	Forward	61.0	Mouse	100
	5'-GGGTTTCCAACGCCCAAAGGAAAT-3'	Reverse	60.5	Mouse	
CD38	5'-CCAAGAACCCTTGCAACATCACA-3'	Forward	58.1	Mouse	117
	5'-GATGGGCCAGGTGTTTGGATTTG-3'	Reverse	58.9	Mouse	
FPR-2	5'-CAACTTTGGATCCTGGGCTCA-3'	Forward	57.4	Mouse	116
	5'-CAATTGACATGGGCATGCTGA-3'	Reverse	56.2	Mouse	
Vimentin	5'-GTGGATCAGCTCACCAACGA-3'	Forward	57.2	Mouse	160
	5'-AAGCATTGTCAACATCCTGTCTG-3'	Reverse	55.6	Mouse	
Fascin	5'-AAGCTGATTAACCGCCCCAT-3'	Forward	57.0	Mouse	157
	5'-TGCCCGTGGAGTCTTTGATG-3'	Reverse	57.6	Mouse	
FGF-2	5'-AGGAGTTGTGTCTATCAAGGG-3'	Forward	53.8	Mouse	125
	5'-TATTAGATTCCAGTCGTTCAAAGA-3'	Reverse	51.6	Mouse	
VEGF	5'-CGAGGCAGCTTGAGTTAA-3'	Forward	52.2	Mouse	135
	5'-GGTGACATGGTTAATCGGTC-3'	Reverse	53.3	Mouse	
WT-1	5'-AAGGATACAGCACGGTCACTTT-3'	Forward	56.5	Mouse	106
	5'-ATGGGGTCCTCGTGTTTGAAG-3'	Reverse	57.3	Mouse	
Tie-2	5'-TTAGTCCCCAGGGAGCAGTACA-3'	Forward	59.4	Mouse	116
	5'-TCTGGTTGAGGAGGGAGAATGTCA-3'	Reverse	59.3	Mouse	

Results

Effect of 1400W on kidney injury during renal ischemia and reperfusion

Kidneys from Balb/c adult mice were subjected to 30 minutes of ischemia and 24 hours of reperfusion. The kidney injury biomarker panel (NGAL, Clusterin, and IGFBP7) was assessed through mRNA expression in the kidney cortex and medulla separately. Compared with sham, I/R increased the NGAL and Clusterin mRNA expression in both kidney sections (cortex and medulla). Interestingly, 1400W decreased the NGAL and Clusterin I/R-upregulation only in the renal cortex but not in the renal medulla (Fig. 1A-B). The insulin-like growth factor-binding protein 7 (IGFBP7), a biomarker of risk of acute kidney injury [36] did not experiment change in the mRNA expression by I/R or 1400W (Fig. 1C). Additionally, we did not see changes in the iNOS mRNA expression by I/R or 1400W in the renal cortex and medulla (Fig. 1D).

To improve the understanding of the effect of 1400W on kidney injury, we performed histology analysis. The I/R protocol did not produce morphological changes in the glomeruli (Fig. 2A). However, I/R provoked acute tubular injury in the renal cortex (Fig. 2B) and medulla (Fig. 2C). Compared with their respective sham group, the renal cortex in I/R mice showed acute tubular injury characterized by epithelial cell necrosis, flattening of the epithelium, and secondary distension leading to ectasia of tubular protein. In contrast, the pharmacological treatment with 1400W prevented these morphological alterations in the renal cortex. Only a thin area of subcapsular necrosis and cytoplasmic thick resorption droplets at proximal segments was observed (Fig. 2B). However, in the renal medulla, 1400W did not prevent extensive cytoplasmic flattening, tubular distension, sloughed epithelial

cells with intratubular debris, and reabsorption of protein droplets at the straight proximal segments (Fig. 2C, arrows). The sham animals with saline or 1400W were normal (Fig. 2A, B, and C).

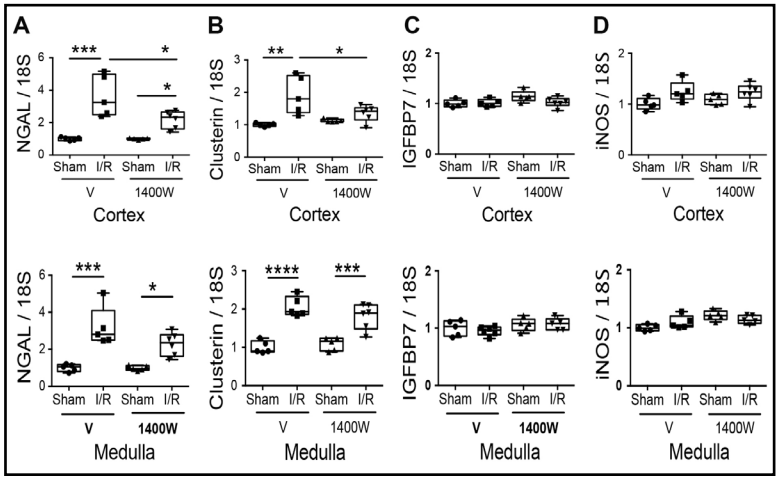

Fig. 1. The I/R-upregulation of kidney injury biomarkers was prevented by 1400W in the renal cortex. Balb/c mice were treated with either vehicle or 1400W (10 mg/kg i.p) before sham or ischemia/reperfusion (30 min of ischemia and 24 hours of reperfusion). The mRNA levels were measurement by qRT-PCR in the renal cortex (upper) and medulla (down) from Sham (n=5), I/R (n= 5), sham + 1400W (n=5), and I/R + 1400W (n=6) in A. Neutrophil gelatinase-associated lipocalin (NGAL). B. Clusterin. C. IGFBP7, and D. iNOS. The boxes represent the interquartile range of the values, whereas the whiskers span represents the minimum to maximum showing all points. The statistical analysis was conducted using the ANOVA and Tukey's post hoc test (significance denoted as *p<0.05).

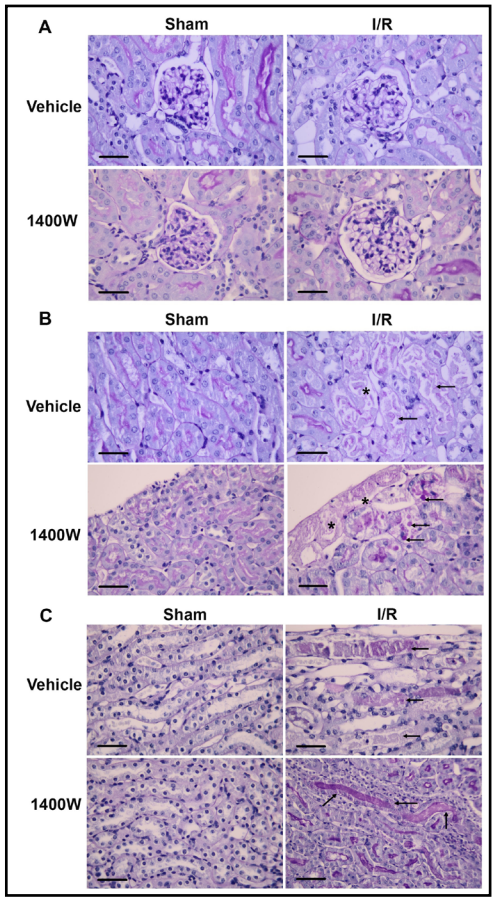

Fig. 2. 1400W reduced the morphological alterations observed by I/R. Balb/c mice were treated with either vehicle or 1400W (10 mg/kg i.p) before sham or ischemia/reperfusion (30 min of ischemia and 24 hours of reperfusion). The tissue damage was evaluated by H/E and periodic acid-Schiff (PAS) staining (only PAS is shown). Representative picture from A. Cortical Glomerulus, B. Cortical tubules, and C. Medullary tubules. Magnification 40X. Scale bar, 50 µm (A, B, C). Asterisk and arrows indicated areas with kidney morphological alterations.

Effect of 1400W on inflammation during renal I/R

It has been widely demonstrated that I/R injury is associated with tubulointerstitial inflammation and exacerbates renal injury [21, 22]. Here, we studied the mRNA expression of a panel of inflammatory cytokines (IL-1β, IL-6, and TNF-α). We observed a significant upregulation of IL-6 and TNF-α mRNA in the renal cortex and medulla compared to the sham group (Fig. 3B-C). Interestingly, the I/R-upregulation of mRNA levels of IL-6 and TNF-α were prevented by 1400W pretreatment in the renal cortex but not in the renal medulla (Fig. 3B-C).

In contrast, the mRNA level of IL-1β was not modified at all (Fig. 3A). In addition, we studied the levels of anti-inflammatory markers (IL-10, Foxp3, and TGF-β). We did not find detectable levels of mRNA expression of IL-10 in the studied groups (data not shown). The Foxp3 mRNA levels were not modified by I/R or 1400W after 24 hours of reperfusion (Fig. 3D). However, the TGF-β mRNA level was upregulated in the renal cortex and medulla by I/R. Interestingly, the 1400W prevented the I/R-upregulation of mRNA levels of TGF-β in the renal cortex but not in the renal medulla (Fig. 3E). TGF-β signaling can induce M2 macrophage polarization in acute damage [37, 38]. Thus, we study the effect of 1400W in the M2 and M1 macrophage polarization in the experimental renal I/R model.

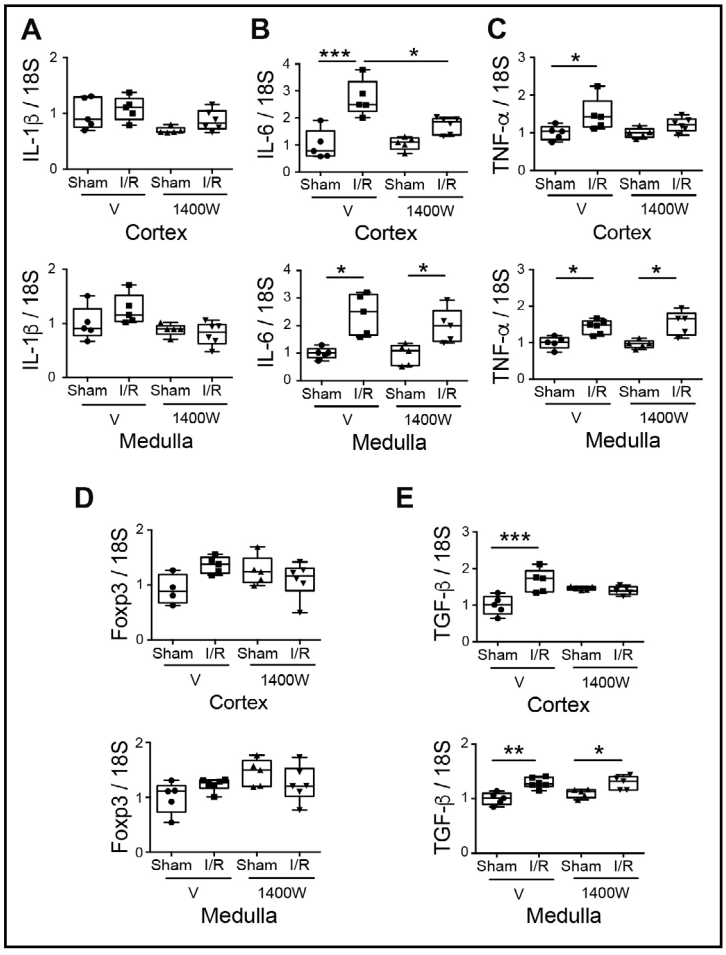

Fig. 3. The I/R-upregulation of inflammation makers was inhibited by 1400W in the renal cortex. Balb/c mice were treated with either vehicle or 1400W (10 mg/kg i.p) before sham or ischemia/reperfusion (30 min of ischemia and 24 hours of reperfusion). The mRNA levels were measurement by qRT-PCR in cortex (upper) and medulla (down) from Sham (n=5), I/R (n= 5), sham + 1400W (n=5), and I/R + 1400W (n=6) in A. IL-1β, B. IL-6, C. TNF-α, D. Foxp-3, and E. TGF-β. The boxes represent the interquartile range of the values, whereas the whiskers span represents the minimum to maximum showing all points. The statistical analysis was conducted using the ANOVA and Tukey's post hoc test (significance denoted as *p<0.05).

Effect of 1400W on macrophage polarization during renal I/R

Macrophage polarization is a critical step in regulating inflammation during renal ischemia [29, 30]. We tested a panel of M2 (ArgI, Erg-2, and c-Myc) and M1 (Fpr-2 and CD38) macrophage markers to study the effect of 1400W in the I/R-induced macrophage polarization. As shown in Fig. 4A-C, the expression of M2 markers was significantly upregulated in the I/R group in both kidney regions (cortex and medulla) (except for ArgI in the renal medulla). The 1400W treatment prevented I/R-upregulation only in the renal cortex and not in the renal medulla. On another side, the M1 marker (Fpr-2, but not CD38) was significantly increased in the I/R mice in the renal cortex and medulla compared to the sham. The treatment with 1400W prevented the I/R-upregulation of Fpr-2 in the renal cortex and medulla. To better understand the effect of 1400W in the balance of M1 and M2 macrophage polarization, we analyzed the M2/M1 ([ArgI*Erg-2*c-Myc]/[Fpr-2*CD38]) ratio in each mouse. We observed that I/R significantly upregulated the M2/M1 ratio in the cortex and medulla. Consistent with the previous findings, the 1400W treatment avoided the I/R-upregulation of the M2/M1 ratio in the cortex but not in the medulla, suggesting that 1400W inhibited the inflammatory response in the renal cortex (Fig. 4F). Interestingly, in the renal medulla, 1400W prevented the M1 polarization without alterations in the M2 polarization of macrophages, suggesting that 1400W did not avoid the promotion of the kidney repair process in the medullary kidney under ischemia and reperfusion injury.

Effect of 1400W on mesenchymal markers expression during renal I/R

Vimentin and fascin-1 are expressed in the mesenchymal phenotype [6, 7]. Here, we found that the mRNA expression of Vimentin was upregulated in the cortex and medulla by I/R (Fig. 5A). Notably, 1400W treatment significantly prevented the renal I/R-induced upregulation of Vimentin only in the renal cortex. In addition, fascin-1 was upregulated only in the kidney medulla section of mice subjected to I/R stimulus, and it was not modified by 1400W treatment (Fig. 5B). These data suggested that 1400W prevented the I/R-upregulation of mesenchymal markers in the renal cortex but not in the renal medulla.

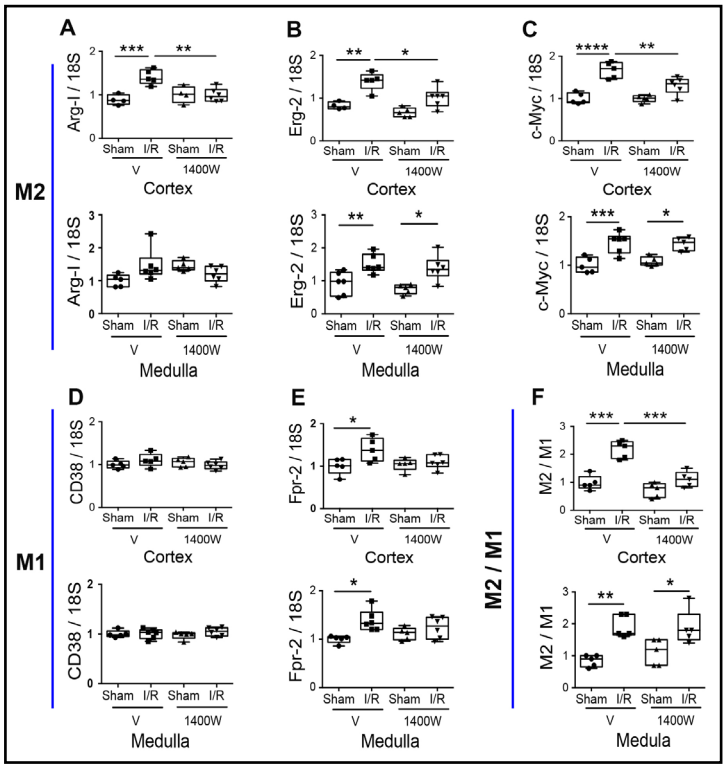

Fig. 4. The I/R-upregulation of the M2/M1 macrophages ratio was avoided by 1400W in the renal cortex. Balb/c mice were treated with either vehicle or 1400W (10 mg/kg i.p) before sham or ischemia/reperfusion (30 min of ischemia and 24 hours of reperfusion). The mRNA levels were measurement by qRT-PCR in cortex (upper) and medulla (down) from Sham (n=5), I/R (n= 5), sham + 1400W (n=5), and I/R + 1400W (n=6). The M2 macrophage markers A. Arg-I, B. Erg-2, and C. c-Myc were assayed. The M1 macrophage markers D. CD38 and E. Frp-2 were measured. F. The M2/M1 ratio was evaluated. The boxes represent the interquartile range of the values, whereas the whiskers span represents the minimum to maximum showing all points. The statistical analysis was carried out using the ANOVA and Tukey's post hoc test (significance denoted as *p<0.05).

Effect of 1400W on nephrogenic gene expression during renal I/R

On another side, Fibroblast Growth Factor 2 (FGF-2) is a protein secreted during kidney development but is less expressed in adult kidney epithelial cells. However, it is reexpressed in response to damage induced by I/R [25, 26] and attenuates I/R Injury via inhibition of endoplasmic reticulum stress [39]. Our results showed that FGF-2 was upregulated in the renal cortex and medulla in animals exposed to I/R damage compared to the respective sham group. The 1400W treatment before I/R significantly decreased FGF-2 expression in the renal cortex but not in the renal medulla (Fig. 6A). Our group previously described the expression of other nephrogenic proteins in tubular cells after kidney damage induced by I/R in rats [25]. They are reexpressed after I/R, such as the vascular endothelial growth factor (VEGF), the angiopoietin receptor (Tie-2), and the Wilms' tumor gene (WT-1), a transcription factor that induces the transformation of mesenchymal cells into metanephrogenic tissue during kidney embryology. In our experimental I/R model, we did not detect changes in the mRNA of these genes after I/R or 1400W treatment with 24 hours of reperfusion (Fig. 6B-D).

Altogether, these results show clear signs of damage, inflammation, macrophage polarization, mesenchymal transition, and nephrogenes reexpression in the renal cortex and medulla induced by I/R injury. Remarkable, the pharmacological inhibition of iNOS using 1400W prevented the I/R-induced kidney alterations in the renal cortex but not in the renal medulla.

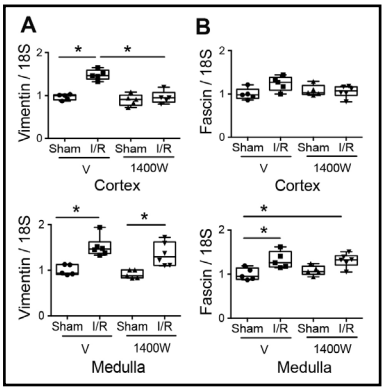

Fig. 5. The I/R-upregulation of Vimentin was counteracted by 1400W in the renal cortex. Balb/c mice were treated with either vehicle or 1400W (10 mg/kg i.p) before sham or ischemia/reperfusion (30 min of ischemia and 24 hours of reperfusion) protocol. The mRNA levels were measurement by qRT-PCR in cortex (upper) and medulla (down) from Sham (n=5), I/R (n= 5), sham + 1400W (n=5), and I/R + 1400W (n=6) in A. Vimentin and B. Fascin-1. The boxes represent the interquartile range of the values, whereas the whiskers span represents the minimum to maximum showing all points. The statistical analysis was conducted using the ANOVA and Tukey's post hoc test (significance denoted as *p<0.05).

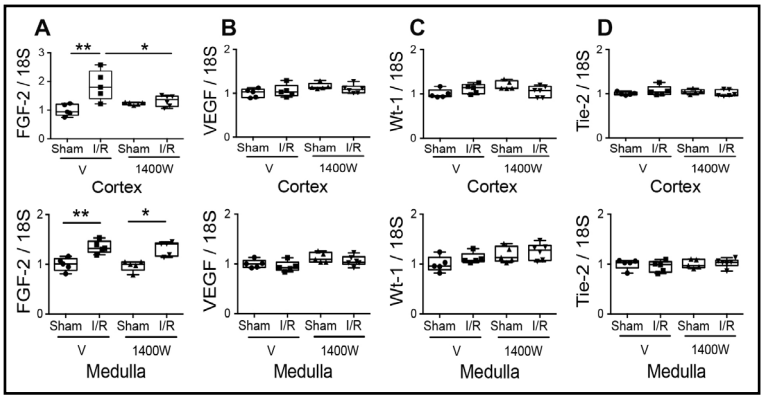

Fig. 6. The I/R-upregulation of FGF-2 was blocked by 1400W in the renal cortex. Balb/c mice were treated with either vehicle or 1400W (10 mg/kg i.p) before sham or ischemia/reperfusion (30 min of ischemia and 24 hours of reperfusion). The mRNA levels were measurement by qRT-PCR in cortex (upper) and medulla (down) from Sham (n=5), I/R (n= 5), sham + 1400W (n=5), and I/R + 1400W (n=6) in A. FGF-2, B. VEGF, C. Wt-1, and D. Tie-2. The boxes represent the interquartile range of the values, whereas the whiskers span represents the minimum to maximum showing all points. The statistical analysis was conducted using the ANOVA and Tukey's post hoc test (significance denoted as *p<0.05).

Discussion

We provided new and interesting evidence about the effects of 1400W on the renal I/R response. In this study, using a mice model of renal I/R, we studied the effects of 1400W on acute kidney injury, inflammation, macrophage polarization, mesenchymal transition, and nephrogenes expression during the first 24 hours of reperfusion. Our results demonstrate that the pharmacological inhibition of iNOS with 1400W affects the renal cortex and medulla distinctly. In the renal cortex, 1400W prevented the I/R-upregulation of 1. NGAL, Clusterin, and signs of morphological damage; 2. IL-6 and TNF-α mRNA; 3. TGF-β; 4. the M2 (Arg1, Erg2, and cMyc) and M1 (CD38 or Fpr2) macrophages makers; 5. Vimentin and FGF-2 mRNA levels. These results indicate that 1400W has a protective effect on the renal cortex. On the other hand, in the renal medulla, we did not see the same effect of 1400W observed in the renal cortex after 24 hours of reperfusion, except for the M1 macrophage marker, Fpr-2, which was downregulated by 1400W in the renal cortex and medulla.

These results clearly indicate that 1400W has a different effect on the renal cortex and medulla. So, the renal medulla showed signs of injury and signal of survivor (M2 macrophage, vimentin, and FGF2) even in the presence of 1400W. In the kidney, after injury by ischemia, repair of the renal epithelium can be elicited via the partial dedifferentiation of differentiated epithelial cells [21], which express mesenchymal markers [25, 26]. FGF-2 is a crucial factor that induces dedifferentiation [40] and participates in the cell regeneration process [41-43].

Our observation provides new findings on the effect of 1400W on macrophage polarization. It is known that M2 macrophages control the renal repair process after I/R [28]. Also, TGF-β promotes M2-like macrophage polarization in THP-1, and murine BMDM cells through SNAIL-PI3K/AKT pathway [37], and the TGF-β signaling pathway controls the expression of genes characteristic for alternatively activated macrophages [38]. Here, in the renal medulla, 1400W reduced the M1 but not the M2 macrophage markers. We observed that renal I/R induced IL-6 and TNF-α mRNA levels in the renal cortex and medulla. However, the IL-1β mRNA was not modified, probably because typical IL-1β activation is mediated by proteolytic casp-1 cleavage after inflammasome formation [44]. Interestingly, 1400W prevented the IL-6 and TNF-α mRNA upregulation only in the renal cortex. Thus, our findings suggest that 1400W prevented the kidney injury in the cortex but lets the reparative mechanism be present in the renal medulla. Our results could be explained by the structural and functional differences between the renal medulla and cortex and the ability of each kidney region to recover from the insults after reperfusion time.

Ischemia and reperfusion injury: cortex vs. medulla

During the renal I/R, the cortical blood flow (CBF) and the medullary blood flow (MBF) decreased dramatically during the ischemic period. During the reperfusion period, the CBF rapidly recovered to baseline levels. In contrast, a transient improvement in MBF was seen immediately after reperfusion, followed by a gradual decline to approximately 50% of original MBF. This prolonged fall in medullary blood flow is associated with a long-term decline in renal function [45, 46]. Evidence indicates that the renal blood flow decrease is more prominent in the outer medulla than in the cortex because the medullary capillaries are more susceptible to vascular congestion accompanying renal ischemia [46]. Thus, if the vascular congestion in the medulla is not relieved, the damage persists [47, 48]. Freitas et al. [49] recently demonstrated (60 minutes ischemia/30-60 reperfusion, rats) that pericyte-mediated constriction, which regulates capillary diameter, may reduce renal blood flow, and physically trap red blood cells extending renal tissue injury following reperfusion [49]. They showed that renal blood flow remained reduced, mainly in the medulla and in minor magnitude in the cortex. Our results showed that 1400W did not prevent kidney damage at the medulla level (NGAL and Clusterin are upregulated) with high levels of TNF-α. Thus, in the renal medulla is probably a persistent vascular inflammation, renal vasoconstriction, and incorrect reperfusion [50].

Also, it is interesting to note that in our model, VEGF, a factor that allows various cell types to survive and proliferate under conditions of extreme stress such as hypoxia [51, 52], was not modified in the cortex or medulla at 24 hours of reperfusion. Similar results were obtained for Tie-2 expression, a factor that promotes angiogenesis. In a rat model of I/R (30 min ischemia), we previously detected VEGF and Tie-2 I/R-upregulation from 24 hours of reperfusion [25]. Moreover, our group demonstrated that when rats were treated with FGF-2, these proteins were upregulated [41]. Most notably, *in vitro* studies have shown that FGF-2 induces the expression of VEGF and contributes to angiogenesis [52]. Here, we observed that 1400W did not prevent the I/R-upregulation of FGF-2 in the renal medulla.

The iNOS inhibitors decrease renal damage after I/R

In the kidney, there are three different isoforms of nitric oxide synthase (NOS): neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS). iNOS is a calcium-independent synthase whose expression is induced by cytokines, oxidative stress, and transcription factor such as NF-κB [53] and contribute to I/R injury in the kidney [6-11]. The NO combines with the superoxide radical and forms the cytotoxic metabolite, peroxynitrite, which causes cell membrane damage through protein nitration with subsequent loss of structure and function [54-56]. Consequently, our previous studies using L-NIL [6] and AG [7] demonstrate that iNOS inhibitors applied i.p before I/R protect the mice kidney from the injury induced by I/R. Here, it showed that 1400W downregulated the kidney injury biomarkers (Clusterin/NGAL) after I/R, attenuating the expression of inflammatory mediators and having a different effect on the renal cortex than the renal medulla. Of course, other signaling pathways could be involved in the effect of iNOS inhibitors.

The 1400W is a competitive inhibitor of iNOS with reported low toxicity and is much more specific than other iNOS inhibitors [12-14]. Thus, 1400W is an irreversible iNOS inhibitor with a K_d value < 7 nM. In contrast, inhibition of human neuronal NOS and endothelial NOS (eNOS) was relatively weaker and rapidly reversible [13]. The 1400W was at least 5000-fold selective for iNOS versus eNOS. Therefore, considering that we use only one dose (10 mg/kg of the animal body weight) and the irreversible iNOS inhibition of 1400W, we believe that the other isoforms were not inhibited. In addition, also previously described that 10 mg/kg of 1400W were efficient in ameliorating experimental renal I/R injury in rat significantly decreasing the NO levels [19]. Therefore, the present study is the first investigation to explore the protective effect of 1400W against I/R separately in the renal cortex and medulla.

Conclusion

In summary, remarkably, we found that 1400W prevented the I/R injury in the renal cortex but not in the medulla (Fig. 7). 1400W treatment reduces the I/R-activation signs of renal damage (tissue morphology, NGAL, and Clusterin expression), mesenchymal transition (vimentin, fascin-1), inflammation, macrophage polarization, and nephrogenic genes only in the renal cortex but not in the renal medulla. Therefore, we think that the action of 1400W could be cell-specific. Consequently, the present investigation provides relevant information to understand that the I/R-drug development must consider different kidney regions to prevent acute kidney injury and dysfunction. The present research showed that 1400W is a good candidate to protect against renal cortex injury and must be combined with a drug to inhibit the renal medulla injury to have an effective therapy against renal I/R injury.

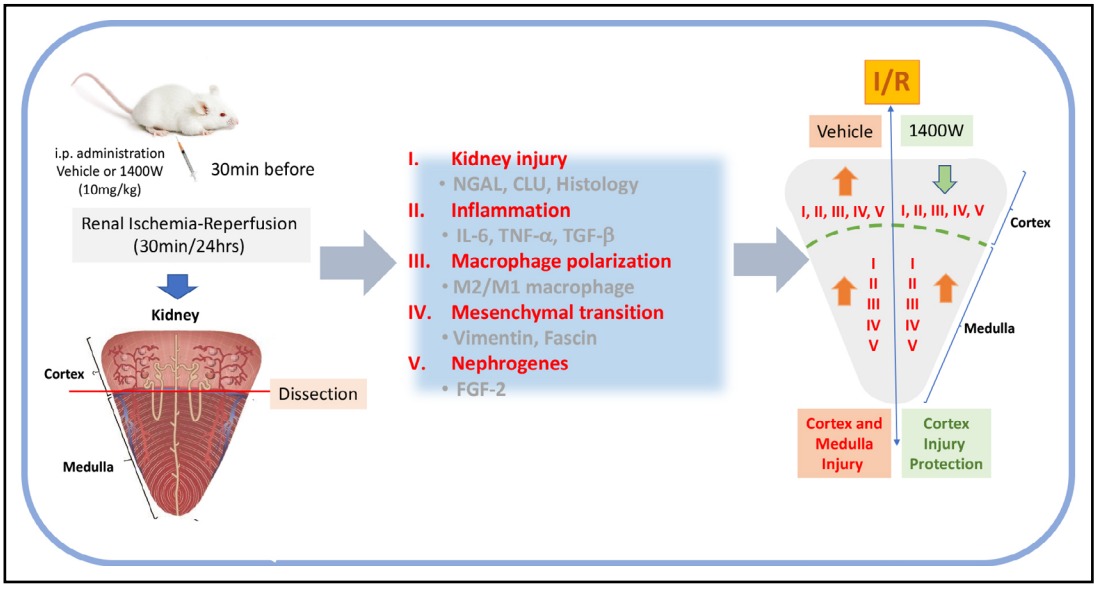

Fig. 7. Schematic representation of events associated with ischemia-reperfusion (I/R) injury and the effect of 1400W. Balb/c mice were treated with either vehicle or 1400W (10 mg/kg i.p) before sham or ischemia/reperfusion (30 min of ischemia and 24 hours of reperfusion). I/R induced alterations in markers associated with different pathways (I-V). 1400W treatment reduced the I/R-activation signs of I) kidney injury (tissue morphology, NGAL, and Clusterin expression), II) inflammation, III) macrophage polarization M2/M1 ratio, IV) mesenchymal transition (vimentin, fascin-1), and V) nephrogenic genes. These effects were only observed in the renal cortex but not in the renal medulla. Thus, 1400W prevents renal injury in the renal cortex but not in the medulla in the murine ischemia and reperfusion injury model.

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Author Contributions

Conceived and designed experiments: CEI, CP. Performed the experiments: CP, ML, GM. Analyzed the data: CP, ML, GM, CEI. Wrote the paper CP, CEI. Contributed reagents/materials/analysis tools: CP, GM, CEI.

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Statement of Ethics

All experimental procedures were under institutional and international standards for humane care and laboratory animal use (Animal Welfare Assurance Publication A5427-01 Office for Protection from Research Risks, Division of Animal Welfare. NIH, USA). All procedures were approved by the Committee on the Ethics of Animal Experiments of the University de los Andes, Chile.

Disclosure Statement

The authors have no conflicts of interest to declare.

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