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Progressive endothelin-1 gene activation initiates chronic/end stage renal disease following experimental ischemic-reperfusion injury

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Abstract

This study assessed whether endothelin-1 (ET1) helps mediate post-ischemic acute kidney injury (AKI) progression to chronic kidney disease (CKD). The impact(s) of potent ETA or ETB receptor-specific antagonists (Atrasentan and BQ-788, respectively) on disease progression were assessed 24 hours or 2 weeks following 30 minutes of unilateral ischemia in CD-1 mice. Unilateral ischemia caused progressive renal ET-1 protein/mRNA increases with concomitant ETA, but not ETB, mRNA elevations. Extensive histone remodeling consistent with gene activation and increased RNA polymerase II binding occurred at the ET-1 gene. Unilateral ischemia produced progressive renal injury as indicated by severe histologic injury and a 40% loss of renal mass. Pre- and post-ischemia or just post-ischemic treatment with Atrasentan conferred dramatic protective effects such as decreased tubule/microvascular injury, normalized tissue lactate, and total preservation of renal mass. Nuclear KI-67 staining was not increased by Atrasentan, implying that increased tubule proliferation was not involved. Conversely, ETB blockade had no protective effect. Thus, our findings provide the first evidence that ET-1 operating through ETA can play a critical role in ischemic AKI progression to CKD. Blockade of ETA provided dramatic protection, indicating the functional significance of these results.

Keywords

Endothelin-1; endothelin-1 receptor A; endothelin-1 receptor B; epigenetic modifications; Atrasentan; BQ-788

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INTRODUCTION

A growing clinical literature indicates that acute kidney injury (AKI) can initiate the onset of progressive renal disease (1-6). For example, in a recent study from Kaiser Permanente of California, it was reported that patients who sustained a bout of dialysis- requiring AKI had a ~28 fold increased risk of developing stage 4 or 5 chronic kidney disease (CKD) (7). However, the mechanisms by which AKI might initiate the onset of CKD have not been defined.

One prominent theory holds that an initial ischemic insult can induce peritubular microvascular damage, thereby compromising renal blood flow (8-10). This may culminate in persistent renal ischemia / hypoxia with ongoing tissue damage. However, the factors that might contribute to this injury pathway have been difficult to define. This is largely due to the fact that the most commonly used model of ischemic AKI, bilateral renal artery occlusion (RAO), does not typically produce progressive renal damage (8-14). Rather, despite the fact that RAO produces so called “healing defects” (e.g., persistent tubular / microvascular damage; salt sensitive hypertension), neither sustained, nor progressive, GFR losses result (8-14).

In contrast to bilateral ischemic injury, we have recently demonstrated that 30 min of unilateral ischemic injury in the mouse produces ongoing tubular necrosis, interstitial inflammation, peritubular microvascular injury, renal fibrosis, and ultimately a 40-50% loss of renal mass over 2-3 weeks (15). Due to the presence of an uninjured contralateral kidney, the natural history of severe AKI can “play out” because the presence of one uninjured kidney prevents death from early uremia. By using this unilateral ischemia model, we have noted several important pathophysiologic events that participate in progressive renal damage. These include stepwise increases in pro-inflammatory cytokine generation, down regulation of selected anti-inflammatory defenses (e.g. heme oxygenase 1 and IL-10), and lipotoxicity (15).

An additional, but as yet unexplored, potential mediator of post-ischemic renal disease progression is the potent vasoconstrictor, endothelin 1 (ET-1; ref. 16,17). In support of its possible role in the pathogenesis of AKI are a few studies in which ET-1 gene deletion, or blockade of the ETA receptor, mitigated the *initiation* phase of ischemic, endotoxemic, or rhabdomyolysis- induced acute renal failure (18-21). However, much conflicting data exist in this regard. For example, at least five studies have found that ET-1 receptor blockade (of either the A or B receptor) either conferred no functional protection, or worsened post-ischemic AKI (22-26). In addition to the unresolved issue of whether ET-1 plays a role in the *initiation* phase of AKI, ET-1’s potential influence on *post- ischemic renal disease progression* has not been addressed. Indeed, if persistent tissue ischemia is a mediator of progressive renal damage, as suggested above, then ET-1- mediated renal vasoconstriction could potentially play a critical pathogenic role.

The goal of the present study was to gain new insights in this regard. Towards this end, we have defined the natural course of ET-1 expression, and that of its A and B receptors, in our unilateral model of progressive post-ischemic renal disease. We then tested whether

blockade of the vasoconstrictive ETA receptor (with ABT-627; “Atrasentan”; 27,28), would confer a protective effect. Conversely, because the ETB receptor has been suggested to induce vasodilation and possible cytoprotective effects (16-18), its potential impact on post-ischemic renal damage was also assessed via pharmacologic ETB selective blockade.

RESULTS

ET-1 mRNA and protein assessments

As shown in Figure 1, within 24 hrs of unilateral ischemic injury, a 4 fold increase in ET-1 mRNA was observed, compared to values in normal kidneys extracted from sham operated controls ($p<0.001$). By 2 weeks post- ischemia, a marked further ET-1 mRNA increase was observed, reaching values that were >10 fold higher than those seen at 24 hrs. These post ischemic ET-1 mRNA increases resulted from ischemia, not surgical stress, given that the contralateral (non ischemic) kidneys retained normal ET-1 mRNA levels.

The marked increase in renal cortical ET-1 mRNA was associated with an approximate 10 fold increase in renal cortical ET-1 protein levels (Figure 2). In contrast, no significant increase in plasma or contralateral kidney ET-1 levels was observed (values remaining close to those seen in either normal mice or in sham operated surgical controls). This implies that the elevated renal cortical ET-1 protein levels in the 2 week post-ischemic kidneys were a result of increased renal ET-1 production, not increased uptake from the systemic circulation.

RNA polymerase II (Pol II) binding and histone modifications at the ET-1 gene

As shown in Fig. 3, the increases in renal cortical ET-1 mRNA at 2 weeks post ischemia were associated with an approximate 5 fold increase in Pol II binding to the start exon of the ET-1 gene, consistent with a marked increase in gene transcription (as assessed by chromatin immunoprecipitation, ChIP, assay). Furthermore, increased levels of each of three assessed ‘gene activating’ histone marks at exon 1 (H3K4m3; H3K9/14Ac, histone variant H2A.Z) corresponded with the increased Pol II levels. Thus, these ChIP data imply that ischemia leads to gene-activating histone modifications at the ET-1 gene, potentially contributing to increased gene transcription via increased Pol II recruitment.

Renal cortical ETA and ETB receptor mRNA expression post ischemia

As shown in Fig. 4, a 3 fold increase in ETA receptor mRNA was apparent by 24 hrs post ischemia. By 2 weeks post ischemia, a further 8 fold increase in ETA receptor mRNA was observed. Thus, compared to basal values, ETA receptor mRNA levels rose ~25 fold over the course of the experiment. In sharp contrast, no increase in ETB receptor mRNA was observed at 24 hrs post ischemia. By 2 weeks post ischemia, an ETB receptor mRNA increase was observed, but it was quantitatively trivial compared to the ETA mRNA increases (2× vs. 25×, respectively).

Unilateral ischemia / renal mass assessments

Control ischemia; Pre + post Atrasentan effects: As shown in the left hand panel of Fig. 5, the unilateral I/R injury protocol induced a ~40% reduction in post-ischemic renal mass

(renal weight), in comparison to the weights of normal kidneys extracted from sham operated mice ($p<0.0001$). Not shown, sham surgery did not independently affect renal weight, compared to the renal weights of normal mice. Administration of Atrasentan, started before renal ischemia and continued throughout the two week post-ischemic period, conferred marked protection, as judged by the fact that the post-ischemic kidney mass (weights) did not significantly differ from that of normal kidneys. A graphic depiction of this result is presented in Fig. 6: the unilateral ischemia / reperfusion (I/R) kidney (far left) was markedly reduced in size, compared to a normal kidney (far right). In contrast, the pre + post ischemia Atrasentan treated kidney (I/R +Atra) manifested essentially normal kidney size.

Post ischemic Atrasentan treatment: To assess whether the above protective effect was mediated by Atrasentan acting in the pre vs. the post- ischemic injury phase, mice were treated with the drug starting 24 hrs post renal ischemia and continued for two weeks. As shown in the right hand panel of Fig. 5, ischemia without drug treatment once again caused a 40% reduction in renal weight. Post-ischemic Atrasentan treatment completely blocked this loss of renal mass, thereby recapitulating the protection seen in the pre + post Atrasentan treatment experiment. This indicates that Atrasentan mediated its protective effect during the delayed (>24 hrs) post-ischemic period, and not in the immediate ischemic/ reperfusion injury phase (i.e., during ischemia and 24 hrs of reflow).

Renal histologic assessments at 2 weeks post unilateral ischemia ± Atrasentan treatment

To confirm Atrasentan's protective effect against ongoing post- ischemic injury, renal histology was examined in kidneys obtained 2 weeks post ischemia with and without Atrasentan treatment. The unilateral ischemia protocol caused marked proximal tubule dropout, extensive interstitial inflammation, ongoing proximal tubule necrosis, and extensive intratubular cast formation. These changes were observed throughout the renal cortex and outer medullary stripe. Atrasentan treatment caused a marked reduction in each of these changes (see Figure 7, far right panel). Blinded grading of the severity of these changes using a semiquantitative score (1+ to 4+; least to most severe injury observed) revealed a marked diminution in injury scores in the Atrasentan group (control ischemia, 3.4±0.3; Atrasentan, 1.3±0.2; $p<0.01$). Thus, the histologic findings corroborated renal protection, as denoted by the Atrasentan- induced preservation of renal mass.

Potential effects on Atrasentan on renal growth independent of ischemic injury

To ascertain whether Atrasentan treatment might impact renal growth / size independent of an effect on renal ischemic injury, renal weights of the contralateral (non ischemic) kidneys from the above experiment were assessed. The contralateral (CL) kidneys manifested an approximate 25% increase in renal weight, compared to normal kidneys (Fig. 7, left; consistent with renal hypertrophy in response to contralateral ischemic injury; ref. 15). Atrasentan had no effect on this hypertrophic response, given that contralateral kidney weights were essentially identical in the no drug vs. drug treatment groups. As shown in the middle of Fig. 7, immunohistochemical staining for KI-67 (a nuclear proliferation marker) demonstrated a marked increase in renal tubular cell proliferation in the 2 week post ischemic kidneys (panel B; see arrow), compared to normal (panel A) kidneys. Given the

marked decrease in renal tubular injury in the Atrasentan treated group (and hence, less of a stimulus for renal regeneration), less KI-67 staining was observed in the post- ischemic kidneys from the Atrasentan treated vs. control ischemic mice. However, within areas in which renal tubular injury was observed in these Atrasentan treated kidneys, increased KI-67 nuclear staining was observed (see arrow at right of panel C).

Exploration of potential Atrasentan effects on the induction phase of ischemic AKI

To further explore the possibility that Atrasentan might mitigate the acute injury phase, and thus, cause a subsequent preservation of renal mass, mice were pre-treated × 24 hrs with the drug and then subjected to bilateral ischemic injury. By so doing, a possible protective effect against acute ischemia could be assessed by potential reductions in post-ischemic BUN and plasma creatinine concentrations. However, as shown in Fig. 8, no protection was observed against either 22.5 min or 25 min bilateral ischemic challenges, as gauged by either BUN or plasma creatinine concentrations. As an additional marker of renal injury, renal cortical NGAL mRNA levels were also assessed. Both I/R protocols induced marked NGAL mRNA elevations (Fig. 8, right panel). However, in neither instance did Atrasentan decrease these NGAL mRNA increases, further supporting the conclusion that Atrasentan was unable to block the initial ischemic injury phase.

Assessment of Atrasentan treatment on post- ischemic lactate levels and CD-34 staining

Unilateral ischemia was associated with an increase in renal cortical lactate levels from 1-3 days post ischemia (see Figure 9, left). With Atrasentan treatment, post-ischemic lactate concentrations remained at or near control levels (NS vs. control values). Thus, these data are consistent with the hypothesis that ETA receptor blockade improved post-ischemic tissue oxygenation, presumably via through an improvement in renal microcirculatory perfusion. Morphologic support for this assumption was apparent from CD-34 staining of the two week post- ischemic kidneys with and without Atrasentan treatment. Marked micovascular dilatation, consistent with vascular stasis/vascular injury, was apparent in control post- ischemic kidneys, as assessed by CD-34 staining. These changes were almost completely absent in the presence of Atrasentan treatment (Fig. 9, right).

ETB receptor blockade with BQ-788

BQ-788 failed to decrease the severity of renal injury, as assessed by loss of renal mass at the two week time point (normal weights, 0.26±0.01 gms; control ischemia, 0.16±0.01 grams; ischemia + BQ-788, 0.16 ±0.03 gms). This was not due to a lack of BQ-788 biologic activity, given that the agent caused an expected compensatory increase in ETB mRNA levels due to ETB receptor blockade (2.6±0.4 vs. 4.6±0.5, control ischemia vs. BQ-788 treated ischemia; $p<0.04$). Thus these findings point to ET-1 / Atrasentan effects on post ischemic renal injury as being mediated through the ETA receptor.

DISCUSSION

Although there have been conflicting suggestions that ET-1 may play a role in the *induction* phase of ischemic AKI (18-26), its potential effects on post- ischemic *disease progression* have not been previously assessed. Given the growing evidence that post-ischemic AKI can

initiate CKD, if ET-1 were to play a pathogenic role, the clinical availability of ET-1 receptor antagonists would imply that therapeutic options are ‘at hand’. Hence, the current study has evaluated the expression of ET-1 and that of its two dominant receptors (ETA, ETB) in our unilateral model of post- ischemic AKI that leads to near ‘end stage’ kidney disease in a matter of weeks.

To gain an initial insight into a possible role for ET-1 in post- ischemic disease progression, renal cortical ET-1 gene expression was assessed. Modest ET-1 mRNA increases were noted by 24 post ischemia. However, a subsequent and seemingly exponential ET-1 response ensued over the next 2 weeks. Thus, by the end of the experiments, ~40-50 fold increases in renal cortical ET-1 mRNA, and 10 fold ET-1 protein elevations, were observed. Notably, despite the dramatic increase in renal cortical ET-1 protein levels, plasma and contralateral kidney ET-1 protein concentrations remained unchanged. This indicates that the post-ischemic renal cortical ET-1 protein elevations reflected intra-renal generation, not uptake from the systemic circulation. Notably, ETA receptor mRNA levels paralleled the rising ET-1 mRNA and protein concentrations. Conversely, relatively trivial, or no, ETB receptor mRNA increases were observed. It is notable that the ETA receptor mediates ET-1’s vasoconstrictive effects, whereas the ETB receptor is believed to exert counter-regulatory vasodilation and cytoprotective actions (16-18). Thus, the preferential increase in the ETA vs. the ETB receptor implies a ‘tipping of the balance” towards ET-1’s potent vasoconstrictive, and hence “injury-provoking”, effects.

Following ischemic renal injury, histone modifying enzyme systems can be activated and induce chromatin remodeling at pro-inflammatory genes (29-32). These changes include histone H3 trimethylation, acetylation, and histone H2A.Z exchange. By loosening chromatin structure, they facilitate RNA polymerase II (Pol II) binding to affected genes and, thus, enhance gene transcription rates. To assess whether such changes could potentially contribute to the progressive activation of the ET-1 gene post ischemia, ChIP assay was applied to 2 week post-ischemic kidney samples. Dramatic increases in all three assessed ‘gene activating’ histone marks (H3K4m3, H3K9/14 acetylation; H2A.Z) were observed. The functional significance of these changes was implied by parallel increases in the binding of Pol II (the enzyme that drives transcription) to the ET-1 gene. While definitive cause -and- effect relationships between these histone modifications and increased ET-1 gene transcription rates remain to be proven, that these histone changes, are indeed, ‘gene activating’ in a variety of biologic systems certainly suggests that this is the case (29-32).

Atrasentan is a highly potent and selective ETA receptor antagonist (~1,800 fold greater specificity for ETA vs. ETB; 27, 28). Given the dramatic increases in intrarenal ET-1 protein levels, and given the 25 fold increases in ETA receptor mRNA, we assessed whether blockade of ET-1 - ETA receptor binding would mitigate the seemingly inexorable progression of post-ischemic unilateral renal disease. Indeed, this was the case: pre + post ischemic Atrasentan administration conferred virtually complete protection against post-ischemic reductions in renal mass (40% vs. 0% renal mass loss without vs. with Atrasentan therapy). That marked histologic protection was also observed underscores Atrasentan’s protective effect. To discern whether this protection was exerted against the initial injury

phase, thereby culminating in less 'downstream' renal damage, or whether Atrastentan's protective effect was induced during the post-ischemic phase, the drug was started 24 hrs after the initial ischemic event. Again, complete preservation of renal mass was observed (i.e., equal to that seen with combined pre + post Atrasentan treatment). Further supporting the conclusion that Atrasentan protected only during the post-ischemic period was indicated by two additional observations: *first*, the drug was unable to mitigate the early (0-24 hrs) phase of post- ischemic AKI, as assessed by 24 hr BUN, plasma creatinine, and NGAL mRNA levels; and *second*, in data not shown, Atrasentan failed to mitigate ATP depletion injury (as induced by antimycin / 2-deoxyglucose) in cultured proximal tubule (HK-2) cells (33,34). Indeed, given that Atrasentan was able to block evolving renal injury when started well beyond the initial injury phase could have substantial clinical relevance. Because the vast majority of AKI patients are seen following the establishment of ARF, not prior to it, identifying an agent can prevent post- ischemic disease progression suggests potential therapeutic application.

Because ET-1 has potential anti-mitogenic influences (35-37), a theoretical consideration is whether Atrasentan might slow renal regeneration in the aftermath of ischemic AKI. If so, this could potentially represent an adverse effect. To test for this possibility, degrees of renal hypertrophy/hyperplasia were assessed in contralateral kidneys obtained from the unilateral ischemia experiments ± Atrasentan treatment. As shown in Fig. 7, an approximate 25% increase in contralateral (right) kidney weight was observed 2 weeks after left ischemic renal damage, and this result was unaffected by Atrasentan treatment. To further explore this issue, renal immunohistochemical staining for KI-67, a nuclear protein marker of all active cell cycle phases (G_1, S, G_2, mitosis; but not G_o), was assessed (38). As expected, a marked increase in KI-67 nuclear staining was observed in control post- ischemic kidneys, compared to normal renal tissues. Although less KI-67 staining was seen in post- ischemic Atrasentan treated kidneys, this was almost certainly due to the lesser degree of overall tubular damage, presumably leading to a less robust renal tubular proliferative response.

It should be noted that Atrasentan- mediated ETA blockade could potentially confer renal protection not strictly by blocking downstream ETA receptor signaling, but also, by increasing ET-1 availability to the unblocked ETB receptor. Noteworthy in this regard is that ET-1 / ETB signaling can exert cytoprotective effects (16-18). If the latter were relevant to the post-ischemic kidney, then the administration of an ETB receptor antagonist would be expected to worsen post- ischemic renal damage. To test for this possibility, the impact of a potent ETB receptor antagonist, BQ-788, on post- ischemic renal injury was assessed, and no change in disease progression / loss of renal mass was observed. Indeed, these results might be expected, given that ischemia / reperfusion had a relatively trivial effect on ETB receptor expression, at least as assessed by levels of its mRNA. Thus, these experiments underscore the primacy of the ETA receptor in the observed post- ischemic progressive renal disease.

To gain some support for the concept that ETA blockade conferred protection via an improvement in the renal microcirculation, renal cortical lactate concentrations were assessed from 1-3 days post ischemia in the presence and absence of Atrasentan treatment. As shown in Fig. 9, Atrasentan almost completely normalized the elevated tissue lactate

levels during this period, clearly suggesting that decreased vasoconstriction, with improved oxygen delivery, were operative during this period. Furthermore, at two weeks post ischemia, remarkably less microvascular injury / vascular congestion was observed with Atrasentan treatment, as assessed by CD-34 staining. In concert, these two observations strongly point to improvements in the renal microcirculation with Atrasentan, as would be expected from an ETA blocking agent. However, it should be noted that other ET-1 / ETA sensitive injury pathways (e.g., nitric oxide signaling, the angiotensin II system, and TGF-β mediated fibrosis) could also have been involved; e.g. ref. 39). Thus, future studies will be required to dissect out the relative hemodynamic vs. non hemodynamic pathways through which ETA blockade confers its dramatic post-ischemic cytoprotective effect.

Finally, a number of queries arise from consideration of the above experiments. The first is that Yang et al have suggested that a dominant mechanism by which unilateral ischemia produces progressive renal damage and fibrosis is via the development of G2/M growth arrest (40). However, that ETA blockade completely prevented a loss of renal mass implies that this previously observed growth arrest (40) is in fact induced by ongoing ET-1 mediated renal vasoconstriction / tissue hypoxia, and resultant tissue damage. A second question that arises from the current experiments is why equal degrees of contralateral kidney hypertrophy developed whether or not ETA blockade was induced. Indeed, the Atrasentan-induced preservation of post-ischemic renal mass might be expected to decrease compensatory hypertrophy in the contralateral kidneys. These findings suggest that the stimulus for renal hypertrophy is expressed in the early post ischemic period, prior to the emergence of Atrasentan's ultimate protective effects. The third question raised by the current study is whether some of Atrasentan's protective effects could have been mediated by changes in systemic blood pressure, rather than changes in intrarenal hemodynamics. However, in studies not presented (for space considerations), we observed that our unilateral ischemia model does not raise systemic mean arterial blood pressure (MAP; as assessed with tail blood pressure monitoring), and Atrasentan did not induce significant MAP reductions (86 vs. 82 mm Hg without and with ETA blockade, respectively; assessed over a one week time frame). This implies that it is Atrasentan's intrarenal, not potential systemic, hemodynamic effects that induced its protective effects.

In conclusion, the present study demonstrates that post-ischemic renal injury is associated with a marked and progressive activation of the ET-1 gene, as denoted by rising ET-1 mRNA and protein levels, and a 5 fold increase in Pol II binding to the ET-1 gene. These changes are associated with extensive 'gene activating' chromatin remodeling at the ET-1 gene, suggesting that epigenetic alterations likely facilitate post- ischemic ET-1 gene transcription. Paralleling these changes are preferential increases in post- ischemic ETA (vs. ETB) receptor expression, further suggesting that increased ET1 - ETA receptor signaling likely occurs. Functional significance of these changes to post- ischemic disease progression is indicated by the fact that a highly potent and specific ETA receptor antagonist, Atrasentan, but not an ETB receptor antagonist (BQ-788), completely prevented an otherwise 40% loss of post- ischemic renal mass. This protective action is expressed in the post- ischemic injury phase, given that delaying therapy for 24 hrs post ischemia did not detract from the agent's renal sparing effects. While multiple protective mechanisms could potentially be operative, an improvement in the post-ischemic microcirculation /

microvascular integrity seem likely to be involved. This assertion is based on Atrasentan's ability to markedly diminish post-ischemic tissue lactate levels, and to decrease microvascular injury/congestion, based on CD-34 vascular assessments. Finally, we believe that this is the first study of its kind to demonstrate that any pharmacologic agent can confer essentially complete protection against AKI progression to chronic / end stage renal disease. Thus, the current results would appear to be important, both from an understanding of underlying pathophysiologic mechanisms as well as potential clinical relevance, given that ETA blocking agents are currently 'at hand'.

METHODS

All experiments were performed using male 30-45 gram CD-1 mice, obtained from Charles River Laboratories, Wilmington, MA. They were housed under routine vivarium conditions with free food and water access. Surgeries were conducted under deep pentobarbital anesthesia (40-50 mg/Kg IP). Post-surgical analgesia was provided with buprenorphine (0.1 mg/Kg IM). All surgical procedures were approved by the institution's IACUC, in accordance with NIH guidelines.

Quantifying ET-1 and ETA / ETB receptor expression following ischemic renal injury

Ten mice were subjected to a midline laparotomy, and the left renal pedicles were exposed and occluded × 30 min using atraumatic microvascular clamps. Body temperature was maintained at 37°C with an external heating source. After vascular clamp removal, uniform reperfusion was confirmed by loss of kidney cyanosis. The abdominal incision was then sutured in two layers, and the mice were allowed to recover from anesthesia. Ten sham operated mice served as controls.

At either 24 hrs or 2 weeks post surgery, half of the mice in the post unilateral ischemic group (N, 5) or the sham- operated group (N, 5) were re-anesthetized and the abdominal incisions were opened. A blood sample was obtained from the inferior vena cava and both kidneys were resected. They were iced, and the renal cortical samples were cut with a razor blade and extracted for RNA (RNeasy; Qiagen), and total protein. RNA samples were used to determine the mRNAs for ET-1 and its A (ETA) and B (ETB) receptors by competitive RT-PCR using the primers shown in Table 1. Results were expressed as ratios to simultaneously obtained GAPDH product, used as a 'housekeeping' gene. ET-1 protein concentrations in renal cortical extracts and plasma were determined by ELISA (Enzo Life Sciences, Farmingdale, NY).

Chromatin immunoprecipitation assay (ChIP): ET-1 chromatin remodeling and RNA polymerase II (Pol II) binding

Renal cortical chromatin extracts were prepared from the following kidneys: kidneys from three sham operated mice (2 weeks post surgery); three 2 week post ischemic kidneys; and three corresponding contralateral kidneys. Using ChIP assay, degrees of Pol II binding, histone H3 trimethylation (H3K4m3), histone H3 acetylation (H3K9/K14), and Pol II levels at exon 1 of the ET-1 gene were assessed by real time PCR (29-32). In addition, the degree of histone H2A.Z variant exchange at ET-1 exon 1 was assessed (29-32). Results were

expressed as the amount of Pol II, H3K4m3, H3K9/14 Ac, and H2A.Z at ET-1 exon 1 / per mg of probed chromatin protein.

Post- ischemic renal disease progression: impact of ETA receptor blockade

Eighteen mice were subjected to the above unilateral ischemic injury protocol. Nine of the mice received the highly potent and specific ETA receptor antagonist ABT-627 (Atrasentan; ref. 27,28). The Atrasentan was administered in the drinking water (25 μg/mL; designed to equate with a dose of ~5 mg/Kg/day). The drug was started one day before surgery and continued throughout the remainder of the 2 week experiment. Fresh drug was provided 2-3x per week. The remaining 9 mice received only free food and water access, serving as controls.

Upon completion of a two week post- ischemic recovery period, the mice were re-anesthetized with pentobarbital, the abdominal incision was re-opened, and the left (post ischemic) kidneys and the right (contralateral) kidneys were removed and weighed. The degree of post- ischemic loss of renal mass was assessed by comparing the weights of kidneys from sham operated mice, control post- ischemic mice, and post- ischemic mice that had received Atrasentan treatment. Finally, frontal sections of post- ischemic kidneys were taken from 5 control mice and 5 Atrasentan treated mice, fixed in 10% buffered formalin, and used for subsequent histochemical analyses.

Renal histology—*Two* micron sections were cut and stained with hematoxylin and eosin for overall assessment of the severity of tissue injury. The severity of histologic injury was assessed by blinded scoring of slides of 2 week post-ischemic kidneys from 5 Atrasentan treated mice (pre + post ischemia treatment), and from 5 non Atrasentan treated post ischemic controls (semiquantitative scale of 1+ to 4+, or least to most severe renal injury observed, based on the extent of proximal tubule dropout / necrosis and cast formation). In addition, renal tubular cell proliferation was assessed by immunohistochemical staining for KI-67, a nuclear protein marker of all active cell cycle phases (G1, S, G2, mitosis; but not Go) (38).]. Finally, renal microvascular integrity was assessed by immunohistochemical staining for the endothelial cell marker, CD-34 (38). [Note: at 2 weeks post unilateral ischemia in the absence of Atrasentan treatment, modest interstitial collagen deposition is apparent both by Sirius red and Masson Trichrome staining (15, 38). Given the almost normal renal histology with Atrasentan treatment; see Results, collagen deposition was not assessed in Atrasentan treated kidneys].

Determination of whether Atrasentan treatment, restricted to the post- ischemic period, confers renal protection

The above experiment was repeated, but Atrasentan administration was commenced *24 hrs after the induction of ischemic damage*. At the end of two weeks, the mice were re-anesthetized and the left and right kidneys were weighed. The degree of post- ischemic renal weight reduction (the primary endpoint of the above described experiment) was determined. As above, the values were contrasted between the unilateral ischemic kidneys ± Atrasentan treatment (n, 4 each group), and to values obtained in 5 kidneys obtained from normal mice.

Determination of whether Atrasentan pre-treatment protects against the acute ischemic injury phase

Nine mice were pre-treated for 24 hrs with Atrasentan and then they were subjected to either 22.5 min (n, 6) or 25 min (n, 3) of bilateral ischemic injury. An equal number of mice were subjected to the same bilateral ischemia protocols without drug treatment. Atrasentan was continued during the post- ischemic period. Twenty four hrs later, the mice were re-anesthetized, the abdominal incisions were re-opened, a blood sample was obtained from the inferior vena cava, and the kidneys were resected. The ability of Atrasentan to protect against renal ischemia was assessed by determining plasma BUN and creatinine concentrations and by levels of renal cortical NGAL mRNA, a marker of AKI severity (41).

Assessment of Atrasentan treatment on post-ischemic lactate levels

Eighteen mice were subjected to unilateral renal ischemia, half with and half without receiving Atrasentan treatment. Either 24 hrs, 48 hrs, or 72 hrs later, three Atrasentan treated mice and three control ischemic mice were sacrificed, the left post-ischemic kidneys were harvested, renal cortical tissues were extracted and deproteinated, and assayed for tissue lactate levels (Biovision; K607-100; Milpitas, CA). Results were compared to those determined in 4 normal mice and expressed as μmol/mg extracted cortical tissue.

Effect of BQ-788 on post-ischemic renal injury

Eight mice were subjected to the unilateral ischemic injury protocol, with half of the mice receiving the ETB specific receptor antagonist BQ-788. The agent was administered in drinking water (6.7 μg/mL), to provide a dose of ~1 mg/Kg/day (in excess of the K_1 of ET-1 / ETB binding affinity; 42-44). Two weeks post- ischemia, the kidneys were harvested and weighed, as noted above. To confirm BQ-788's biologic activity, levels of ETB receptor mRNA was also assessed (i.e., blockade would be expected to raise ETB mRNA levels).

Calculations and Statistics

All values are presented as means ± 1 SEM. Statistical comparisons were performed by unpaired Student's t test. Continuous variable results were compared by Student's T test. The histologic data were judged by Wilcoxon rank sum test. Significance was judge by a p value of <0.05.

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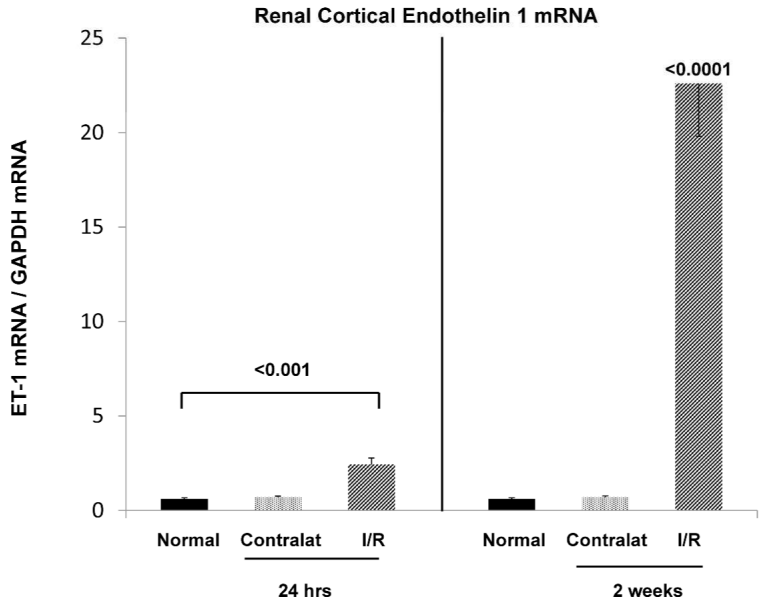

Figure 1. Renal cortical and plasma endothelin 1 (ET-1) mRNA levels following ischemic injury

ET-1 mRNA rose approximately 3 fold by 24 hrs post induction of unilateral ischemic injury, and by 2 weeks, marked further elevations were observed. The specificity of this change for the ischemia- injured kidney was indicated by the normal ET-1 mRNA levels in the contralateral (contralat) kidneys at both the 24 hr and 2 week time points (compared to normal kidney values).

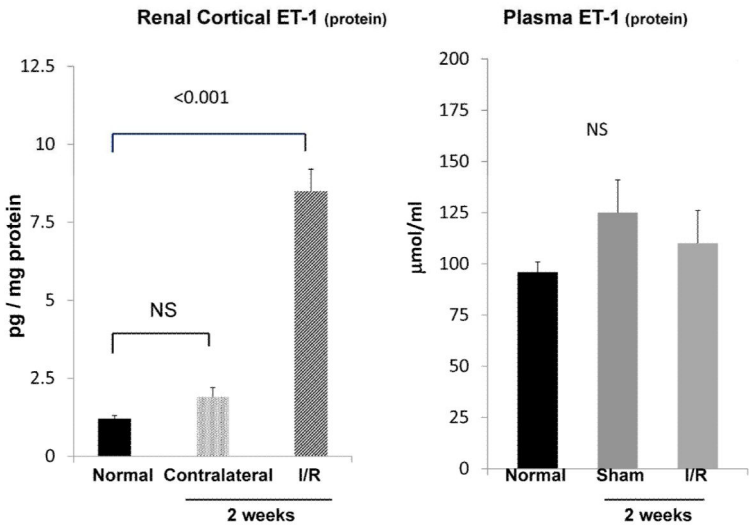

Figure 2. Renal cortical and plasma ET-1 protein levels at 2 weeks post- ischemic injury

Renal cortical ET-1 protein levels were markedly elevated at 2 weeks post ischemia, compared to either normal or contralateral (non ischemic) kidney values (left panel). Conversely, no increase in plasma ET-1 values (right panel) was observed at this time (compared to values in normal plasma or in plasma from 2 week post sham operated mice.)

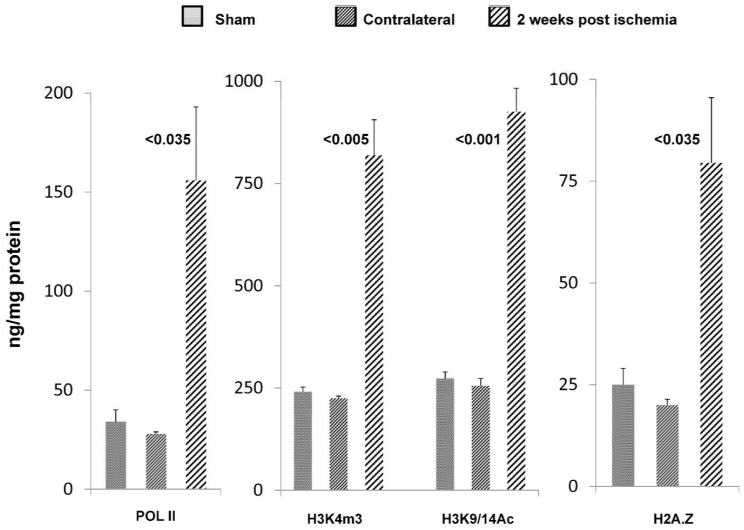

Figure 3. ChIP assay assessments of Pol II binding, histone 3 methylation, histone 3 acetylation, and histone variant H2A.Z at exon 1 of the ET-1 gene

Marked increases in Pol II binding were observed at the ET-1 gene, paralleling the increases in ET-1 mRNA as shown in Fig 1. The increases in Pol II were paralleled by comparable increases in H3K4m3, H3K9/14Ac, and H2A.Z, implying that these 'gene activating' histone marks may have functionally contributed to increased ET-1 gene transcription.

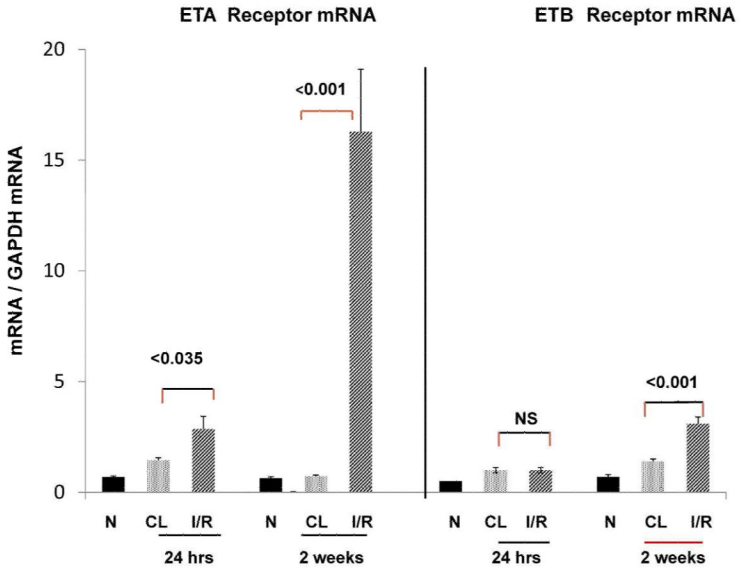

Figure 4. Renal cortical ETA and ETB receptor mRNA levels at 24 hrs and 2 weeks post ischemic injury

ETA receptor mRNA manifested a marked and progressive rise following ischemic injury, reaching values that were ~50 fold higher than the values found in the contralateral (CL) kidney. The latter did not significantly differ from normal (N) kidney values. In contrast, ETB mRNA was not significantly elevated at 24 hrs post ischemia and it rose to only twice the values seen in the non-ischemic contralateral (CL) kidneys. In sum, these data indicate a preferential increase in ETA vs. ETB mRNA expression.

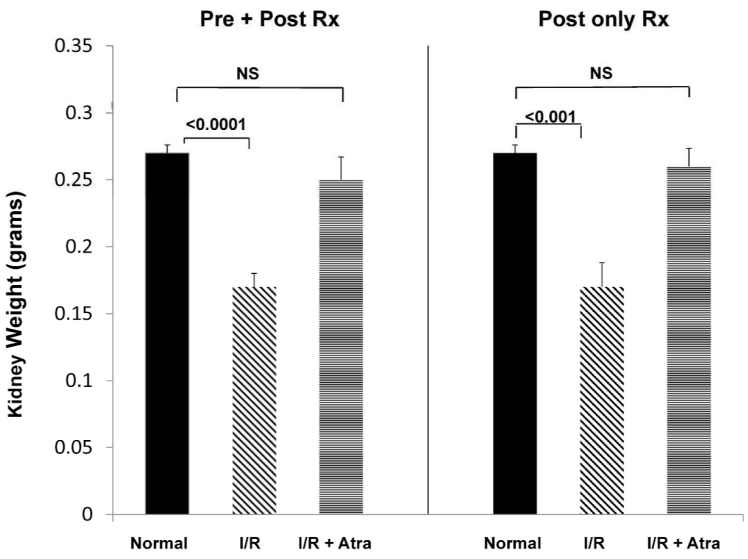

Figure 5. Renal weights 2 weeks after the induction of unilateral ischemic injury +/− Atrasentan (Atra) treatment in the pre + post (left panel), or just the post ischemic period

The ischemia / reperfusion (I/R) protocol produced an approximate 40% reduction in renal weight, compared to kidney weights obtained from age matched normal mice ($p<0.0001$). When Atrasentan was administered starting one day prior to ischemia and continued throughout the two week post ischemic period, renal weight was almost completely preserved (NS, vs. normal kidney weights). This benefit was due to Atra's effect in the post-ischemic period, as evidenced by the fact that starting the agent 24 hrs post ischemia conferred the same degree of protection as did the pre + post administration protocol (NS vs. normal kidney weights).

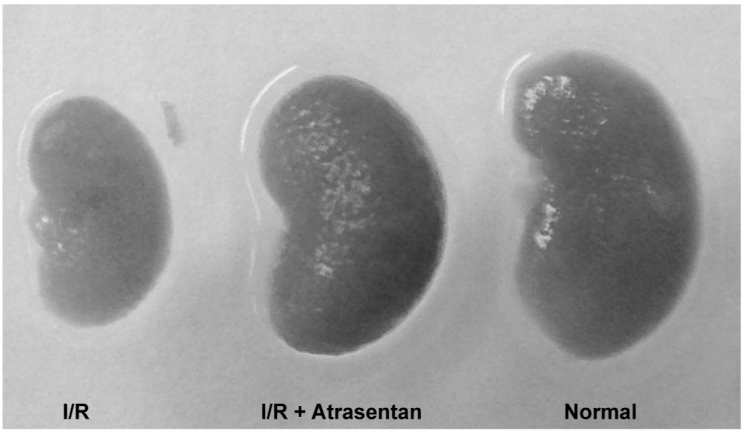

Figure 6. Photographs of a 2 week post- ischemic kidney (left), a post- ischemic kidney with pre + post ischemic Atrasentan treatment (middle), and a normal kidney (right)

This figure graphically depicts the key finding that is presented in Fig 5: i.e., that the I/R protocol induced a dramatic (near 40-50%) reduction in renal size, and that Atra conferred essentially complete protection against the ischemia- induced loss of renal mass.

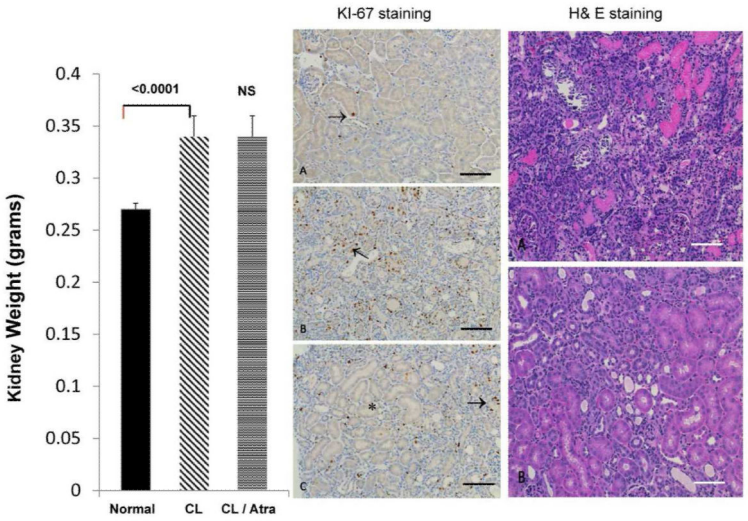

Figure 7. Renal proliferation and histology following the unilateral ischemic protocol with and without Atrasentan treatment

Left hand panel: This panel depicts the weights of the contralateral (CL; non ischemic) kidneys from the unilateral ischemic injury experiments ± Atra treatment. As shown, the CL (right) kidneys manifested compensatory hypertrophy in response to left kidney ischemia, such that by 2 weeks, an approximate 25% increase in renal weight was apparent compared to normal kidneys. Atrasentan did not exert an independent proliferative or anti-proliferative effect, given that the CL kidney weights from the Atrasentan treated group were identical to those seen in the non Atra treated group.

Middle panel. This panel depicts KI-67 staining of a normal kidney (A), a 2 week (left) post ischemic kidney (B), and a 2 week left post ischemic kidney with pre + post Atra treatment (C). The post ischemic kidneys manifested a marked increase in nuclear KI-67 staining, compared to that seen in normal kidneys (arrows point to examples of positive KI-67 nuclear staining). Less KI-67 staining is apparent in the Atrasentan treated kidney, given that less tubular damage, and hence, less stimulus for a regenerative response, would be expected to occur. For example, as depicted, much greater preservation of tubular mass (denoted by asterix) is apparent in the Atra + ischemia vs. the control ischemic kidney section. However, in areas in which cell injury was observed, increased KI-67 staining is observed (arrow in panel C). Scale bar = 80 microns.

Right hand panel. Panel A (top figure) depicts severe tubular dropout, cast formation, and interstitial inflammation at 2 weeks post ischemia following unilateral ischemia. Marked morphologic protection is depicted in a kidney obtained 2 weeks post ischemia with concomitant Atrasentan treatment. Scale bars = 100 micron.

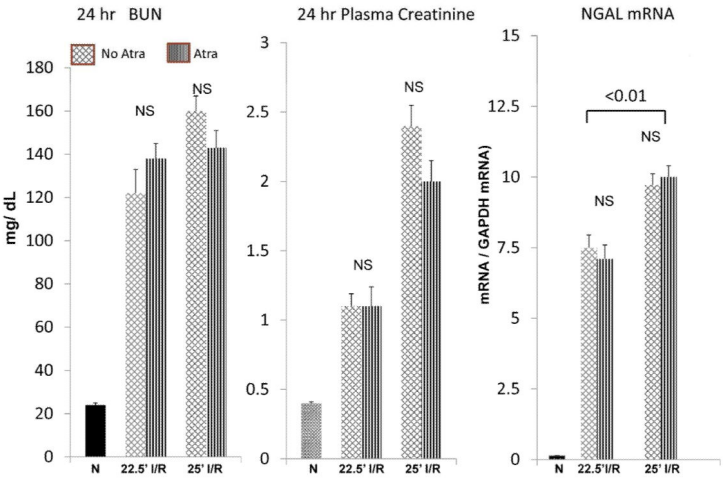

Figure 8. Atrasentan fails to protect against the induction phase of ischemic- reperfusion injury

Mice were subjected to either 22.5 minutes or 25 min of bilateral ischemic renal injury in the presence or absence of Atra pre-treatment for 24 hrs before and for 24 hrs after the induction of renal ischemia. The severity of injury was assessed at 24 hrs post ischemia by BUN and plasma creatinine concentrations and by the extent of induction of renal cortical NGAL mRNA. Atra failed to mitigate AKI severity, as assessed by any of these three parameters.

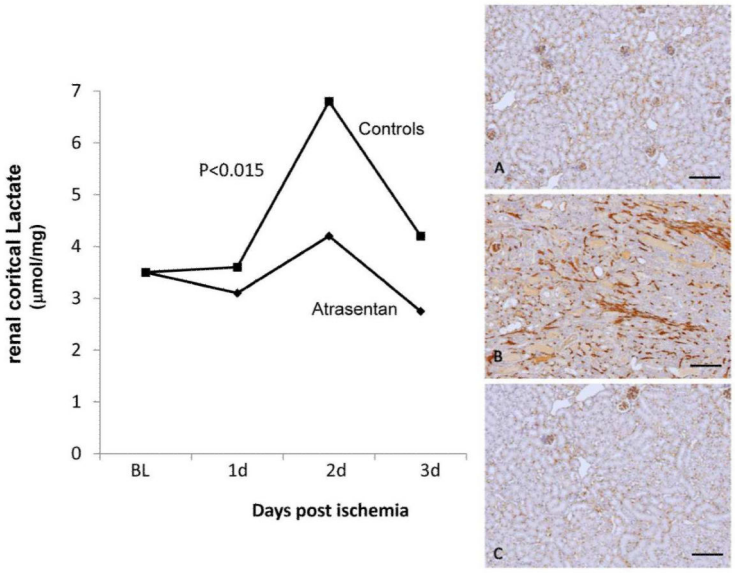

Figure 9. Renal cortical lactate concentrations in normal and post- ischemic kidneys; CD-34 staining of the microvasculature with and without Atrasentan treatment

Renal cortical lactate concentrations were elevated from 1-3 days post ischemia, compared to baseline (BL) values (left panel). Atrasentan almost completely normalized these elevated levels (control vs. Atrasentan treated post-ischemic kidney tissues, $p<0.015$). A morphologic correlate of an improved microcirculation is that control post-ischemic kidneys demonstrated marked microvascular dilatation, consistent with vascular congestion, whereas the microvascular pattern appeared normal at two weeks post- ischemia with Atrasentan treatment. Histologic photomicrographs depict the region of the inner cortex / outer medullary stripe. **A**, normal kidney, **B**, 2 weeks post ischemia; **C**, 2 weeks post ischemia with Atrasentan treatment. Scale bar = 200 micron.

Table 1

Mouse Primers for RT-PCR

Primers used for semiquantitative analysis of endothelin 1 (ET-1), and it's A and B receptors (ETA, ETB, respectively). Products were factored by simultaneously obtained GAPDH product.

mRNA	Primer Sequences	Product Size
ET-1	5′-TCC TCT GCC CGT CTG AAC AAG AAA-3′ 5′-GCC ATC AGC AAT AGC ATC AAG GCA-3′	**239 bp**
ETA receptor	5′-TCC TAT GCA GCT CGC CCT TGT ATT-3′ 5′-ATC ACC GTC TTG AAC CTC TGT GCT-3′	**202 bp**
ETB receptor	5′-CAG TCT TCT GCC TGG TCC TC-3′ 5′-CCA GCA GCA CAA ACA TGA CT -3′	**242 bp**
GAPDH	5′-CTG CCA TTT GCA GTG GCA AAG TGG-3′ 5′-TTG TCA TGG ATG ACC TTG GCC AGG-3′	**437 bp**