Thromboxane Prostanoid Receptors Enhance Contractions, Endothelin-1 and Oxidative Stress in Microvessels From Mice With Chronic Kidney Disease

Cheng Wang, Zaiming Luo, Donald Kohan, Anton Wellstein, Pedro A. Jose, William J. Welch, Christopher S. Wilcox, Dan Wang

Abstract—Cardiovascular disease is frequent in chronic kidney disease and has been related to angiotensin II, endothelin-1 (ET-1), thromboxane A₂, and reactive oxygen species (ROS). Because activation of thromboxane prostanoid receptors (TP-Rs) can generate ROS, which can generate ET-1, we tested the hypothesis that chronic kidney disease induces cyclooxygenase-2 whose products activate TP-Rs to enhance ET-1 and ROS generation and contractions. Mesenteric resistance arterioles were isolated from C57/BL6 or TP-R+/+ and TP-R−/− mice 3 months after SHAM-operation (SHAM) or surgical reduced renal mass (RRM) mice 3 months after SHAM-operation (SHAM) or surgical reduced renal mass (RRM, n=6/group). Microvascular contractions were studied on a wire myograph. Cellular (ethidium: dihydroethidium) and mitochondrial (mitoSOX) ROS were measured by fluorescence microscopy. Mice with RRM had increased excretion of markers of oxidative stress, thromboxane, and microalbumin; increased plasma ET-1; and increased microvascular expression of p22phox, cyclooxygenase-2, TP-Rs, preproendothelin and endothelin-A receptors, and increased arteriolar remodeling. They had increased contractions to U-46,619 (118±3 versus 87±6, P<0.05) and ET-1 (108±5 versus 89±4, P<0.05), which were dependent on cellular and mitochondrial ROS, cyclooxygenase-2, and TP-Rs. RRM doubled the ET-1-induced cellular and mitochondrial ROS generation (P<0.05). TP-R−/− mice with RRM lacked these abnormal structural and functional microvascular responses and lacked the increased systemic and the increased microvascular oxidative stress and circulating ET-1. In conclusion, RRM leads to microvascular remodeling and enhanced ET-1-induced cellular and mitochondrial ROS and contractions that are mediated by cyclooxygenase-2 products activating TP-Rs. Thus, TP-Rs can be upstream from enhanced ET-1, ROS, microvascular remodeling, and contractility and may thereby coordinate vascular dysfunction in chronic kidney disease. (Hypertension. 2015;65:00-00).

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Key Words: cyclooxygenase • mitochondria • oxidative stress • thromboxane • vascular remodeling

Although chronic kidney disease (CKD) is progressive, most patients die from cardiovascular disease (CVD) before reaching end-stage renal disease.¹ CVD in patients with CKD is unusual because it is not clearly associated with hypertension, hypercholesterolemia, or obesity (unless extreme).²⁻⁵ Uncertainty concerning the underlying vascular mechanisms has hampered the development of strategies to prevent the CVD.

Patients with CKD have vascular endothelial dysfunction and oxidative stress that can be detected in CKD stage 1.⁶⁻⁷ These changes can be produced by angiotensin II (ANG II) acting on angiotensin type I receptors, endothelin-1 (ET-1) acting on endothelin type A receptors (ETA-Rs) or cyclooxygenase (COX)-dependent prostaglandins (PGs), and thromboxane A₂ (TxA₂) acting on thromboxane prostanoid receptors (TP-Rs).⁸⁻⁻¹¹ TxA₂ has been implicated in the hypertension and renal vasoconstriction, and glomerulosclerosis of rats with CKD,¹² which has been modeled in mice with surgically reduced renal mass (RRM).¹¹⁻¹⁴ This C57/BL6 mouse model has severe oxidative stress but maintains a normal conscious mean blood pressure measured telemetrically over 3 months and has only a modest reduction in glomerular filtration rate over 3 months.¹⁴ Therefore, it is a convenient model to study early CKD without the confounding effects of hypertension or uremia. Moreover, TP-R−/− mice maintain a normal mean blood pressure measured telemetrically and normal basal phenotype which simplifies interpretation of results with this strain.¹¹⁻¹⁵

Reactive oxygen species (ROS) upregulate COX-2 in the vessel wall.⁹ Moreover, ROS react with nitric oxide (NO) to generate peroxynitrite that irreversibly inactivates prostacyclin

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From the Hypertension, Kidney and Vascular Research Center and Division of Nephrology and Hypertension, Department of Medicine (C.W., Z.L., W.J.W., C.S.W., D.W.) and Department of Oncology, Lombardi Cancer Center (A.W.), Georgetown University, Washington, DC; Department of Nephrology, The Third Hospital of Sun Yat-sen University, Guangzhou, People’s Republic of China (C.W.); Division of Nephrology, Department of Medicine, University of Utah, Salt Lake City (D.K.); and Division of Nephrology, Department of Medicine and Department of Physiology, University of Maryland, Baltimore, MD (P.A.J.).

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Correspondence to Dan Wang, Division of Nephrology and Hypertension, Georgetown University Medical Center, Bldg D, Room 399, 4000 Reservoir Rd NW, Washington, DC 20007. E-mail dw32@georgetown.edu

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Table 1. Basal Parameters, Plasma Endothelin-1 and 24-Hour Urinary Excretion in C57BL/6 Mice: Comparison of Sham Operated and Reduced Renal Mass Mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>SHAM (n=5)</th>
<th>RRM (n=5)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (BWt), (g)</td>
<td>32.6±0.8</td>
<td>33.1±0.9</td>
<td>NS</td>
</tr>
<tr>
<td>Kidney weight/BWt, mg/g</td>
<td>6.6±0.2</td>
<td>7.8±0.3</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Heart weight/BWt, mg/g</td>
<td>4.5±0.2</td>
<td>4.4±0.2</td>
<td>NS</td>
</tr>
<tr>
<td>Aorta weight/BWt, mg/g</td>
<td>0.13±0.007</td>
<td>0.15±0.008</td>
<td>NS</td>
</tr>
<tr>
<td>Vessel lumen CSA (&gt;1000 μm2)</td>
<td>99.6±31.3</td>
<td>75.4±15.0</td>
<td>NS</td>
</tr>
<tr>
<td>Vessel media CSA (&gt;1000 μm2)</td>
<td>12.9±3.7</td>
<td>17.7±2.7</td>
<td>NS</td>
</tr>
<tr>
<td>Plasma creatinine, μmol/L</td>
<td>0.17±0.02</td>
<td>0.27±0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Plasma endothelin-1, pg/mL</td>
<td>1.6±0.3</td>
<td>3.4±0.3</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Urinary 8-isoprostane F2 α (8-Iso)</td>
<td>2.8±0.38</td>
<td>5.1±0.6</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Urinary malondialdehyde/creatinine, ng/mg</td>
<td>48.0±3.4</td>
<td>69.2±6.8</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Urinary TXB2/creatinine, nmol/mg</td>
<td>2.01±0.15</td>
<td>2.52±0.13</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Urinary microalbumin/creatinine, μg/mg</td>
<td>7.3±0.3</td>
<td>12.6±2.0</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

Mean±SEM values as fold relative to β-actin for protein (see Figures S1–S3 for blots) or relative to 18s rRNA for mRNA (see Figure S4). COX indicates cyclooxygenase; endothelin A-R, endothelin type A receptor; NS, nonsignificant; RRM, reduced renal mass; and TXB2, thromboxane B2.

Methods

Animal Preparation and Surgery

Male C57BL/6 mice weighing 25 to 30 g (Charles River Laboratory, Germantown, MD), TP-R knockout (−/−), and TP-R wild-type (TP+/+) mice (C57BL/6J background, kind gift from Thomas Coffman, MD, Duke University, Chapel Hill, NC) were maintained on tap water and standard chow (Na+ content 0.4 g/100 g; Harlan Teklad) and allowed free access to tap water. All of the procedures conformed to the Guide for Care and Use of Laboratory Animals by the National Institute for Health for Laboratory Animal Research and were approved by the Georgetown University Animal Care and Use Committee.

A 2-step surgical 5/6 nephrectomy procedure was used to create RRM under inhalational anesthesia with 2% isoflurane and oxygen mixed with room air in a vaporizer. Approximately, two thirds of the mass of the left kidney was ablated by stitching off each pole using an absorbable hemostat (Ethicon, Inc.). At a second surgery 1 week later, the right kidney was removed. SHAM-operated control mice (SHAM) were subjected to a similar 2-stage procedure without the removal of renal mass. Mice were studied after 3 months, at which time they had considerable oxidative stress with a 7-fold increase in excretion of 8-isoprostane F2α, but an unchanged BP and only mild albuminuria, glomerulosclerosis, tubulointerstitial fibrosis, and a 33% reduction in measured overall glomerular filtration rate.13,14

Measurement of Plasma Endothelin 1 Concentration

Plasma concentrations of ET-1 were measured using a Quantikine ELISA kit (R&D Systems, Minneapolis, MN).

Measurement of Urinary 8-Isoprostane F2 α, Thromboxane B2, Malondialdehyde, Microalbumin, and Creatinine

Mice were housed in metabolic cages (Nalgene Nunc International, Rochester, NY). Urine was collected for 24 hours into tubes containing antibiotics as described.23,24 8-Isoprostane F2α, (Cellbio Labs, Inc., San Diego, CA) and thromboxane B2 (TXB2; QY AEE-Bio, Shanghai, China) in urine were quantified by ELISA after purification, extraction, and measurement of individual recovery by spiking with radiolabelled PGE2 as described and validated previously against gas chromatography/mass spectrometry.24 Malondialdehyde (MDA) was measured by an assay kit (Cayman Chemical Company, Ann Arbor, MI)25 and microalbumin by an ELISA kit (Exocell, Philadelphia, PA). Values were normalized with creatinine, which was measured by a urinary creatinine assay kit (Exocell, Philadelphia, PA; see online-only Data Supplement).

Protein Expression of Mesenteric Resistance Arterioles

The expression of ETA-R, p22phox, p47phox, COX-1 and -2, and TP-R of mesenteric resistance arterioles were quantified using specific antibodies as described13,14,23,25,26 (details see online-only Data Supplement).

RNA Isolation and Real-Time Quantitative RT-PCR

Total RNA isolation and real-time quantitative PCR were performed as previously described with some modifications27 (details see online-only Data Supplement).

Preparation and Study of Mesenteric Resistance Arterioles

Vessels (mean luminal diameter 125±3 mm and length 2 mm) were separated from the superior mesenteric bed, mounted in a wire...
myograph (M610, Danish myotechnology A/S; Aarhus, Denmark), and studied as described (see online-only Data Supplement).

**Experimental Protocol**

The vascular and luminal areas were measured as described. Concentration–response curves to phenylephrine (PE; 10^{-8} to 10^{-5} mol/L, α-adrenoceptor agonist), U-46,619 (10^{-9} to 10^{-6} mol/L, TP-R agonist), and endothelin-1 (ET-1; 10^{-10} to 10^{-7} mol/L) were compared with vehicle. Because PE-mediated contractions were not enhanced in RRM, ET-1 was selected for further studies.

To examine the functional effects of cellular and mitochondrial ROS, vessels were incubated with vehicle, the membrane permeable redox cycling nitroxide tempol (10^{-4} mol/L; Sigma, St. Louis, MO), or the mitochondrial accumulated form MitoTEMPO (10^{-5} mol/L; Sigma, St. Louis, MO) for 30 minutes before testing with ET-1 (10^{-7} mol/L).

To examine the role of PGs and TP-Rs, concentration-dependent responses to ET-1 were obtained after incubation (30 minutes) with either vehicle, SC-560 (10^{-6} mol/L; SC, an inhibitor of COX-1; Sigma, St. Louis), paracoxib (10^{-5} mol/L; Para, an inhibitor of COX-2; Sigma, St. Louis, MO), SC+Para, OKY-046NA (10^{-5} mol/L; an inhibitor of TxA2 synthase [TxA2-S]; Sigma, St. Louis, MO), or SQ-29,548 (10^{-6} mol/L; inhibitor of TP-Rs; Cayman Chemical Company, Ann Arbor, MI). These are fully effective concentrations (see online-only Data Supplement).

**Table 3. Maximum Vascular Contractions and ROS Generation of Mesenteric Arterioles in C57BL/6 Mice: Comparison of Sham-Operated and Reduced Renal Mass Mice**

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>SHAM</th>
<th>RRM</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>PE (10^{-5} mol/L) contraction, %</td>
<td>67.8±3.8</td>
<td>48.1±3.4</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>U-46,619 (10^{-6} mol/L) contraction, %</td>
<td>87.1±5.9</td>
<td>118.0±2.6</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>ET-1 (10^{-7} mol/L) contraction, %</td>
<td>89.1±4.0</td>
<td>108.1±5.1</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>ET-1 (10^{-7} mol/L) cellular ROS generation (Eth/DHE, Δf/f₀)</td>
<td>0.13±0.03</td>
<td>0.33±0.09</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>ET-1 (10^{-7} mol/L) mitochondrial ROS generation (mitoSOX, Δf/f₀)</td>
<td>0.08±0.02</td>
<td>0.17±0.02</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

Mean±SEM values (n=6 per group). ET-1 indicates endothelin-1; PE, phenylephrine; ROS, reactive oxygen species; and RRM, reduced renal mass.

**Figure 1.** Concentration–response relationships for phenylephrine (A), U-46,619 (B), endothelin-1 (C), and cellular and mitochondrial reactive oxygen species generation with 10^{-7} mol/L endothelin-1 (D) in mesenteric resistance arterioles from SHAM (open circles and broken lines or open boxes) and reduced renal mass mice (closed circles and continuous lines or shaded boxes). Comparing groups: *P<0.05; **P<0.01, ***P<0.005. E: Dht indicates ethidium; dihydroethidium fluorescence ratio; and NAK, 125 mmol/L potassium and 10^{-4} mol/L norepinephrine.

Endothelin-1-Stimulated Cellular and Mitochondrial ROS in Mesenteric Resistance Arterioles

ET-1 (10^{-7} mol/L)-induced cellular and mitochondrial ROS production were determined after loading with dihydroethidium or MitoSOX Red as described and fluorescence quantified by PTI RatioMaster™ (Photon Technology International, London, Ontario, Canada; see online-only Data Supplement).

**Prolonged Effect of TP-Rs in Mice With RRM**

RRM or SHAM models were created in TP-R+/+ and TP-R−/− mice and studied 3 months after the surgery. Plasma ET-1, 24 hour urinary 8-Iso, TxB, MDA, microalbumin, mesenteric arteriolar contractions to PE, ET-1, and U-46,619 and generation of cellular and mitochondrial ROS with ET-1 were obtained as described.

**Chemicals and Solutions**

Agents were purchased from Sigma (St. Louis, MI) and dissolved in physiological salt solution.
**Statistical Analysis**

Data are presented as mean±SEM. Cumulative concentration–response experiments were analyzed by nonlinear regression (curve fit) for repeated measurement and differences assessed by 2-way, repeated-measures ANOVA with interaction to assess the effects of RRM versus SHAM, intervention versus vehicle, or TP-R−/− versus TP-R+/+ and the interaction (the effects of the intervention or genotype on the response to RRM). This was followed, if appropriate, with Bonferroni post hoc t-tests for multiple comparisons. A probability value <0.05 was considered statistically significant.

**Results**

**Body, Kidney, and Heart Weights, Mesenteric Resistance Arteriole Remodeling, Plasma Endothelin-1, and Renal Excretion of Biomarkers**

Mice with RRM had normal body, heart, and aorta weights, but despite removal of two-thirds of the left kidney to create RRM, its mass at 3 months exceeded that of SHAM mice, as reported previously (Table 1). Mice with RRM had an increased media:lumen ratio of mesenteric resistance arterioles and increased plasma ET-1 and renal excretions of 8-Iso, MDA, TxB2, and microalbumin (P<0.05).

**Protein Expression in Mesenteric Resistance Arterioles**

Vessels from mice with RRM had increased protein expression of ETA-R, p22phox, COX-2, and TP-R but no significant change in p47phox or COX-1 (Table 2; Figures S1–S3 in the online-only Data Supplement).

**Gene Expression in Mesenteric Resistance Arterioles**

Vessels from mice with RRM had increased mRNA expression of ETA-R, preproendothelin-1, p22phox, and COX-2 but no significant change in COX-1 or TP-R (Table 2; Figure S4).

**Table 4. Effects of Metabolism of Cellular or Mitochondrial ROS or Antagonist of Cyclooxygenase-1 or -2, Thromboxane A2 Synthase or Thromboxane Prostanoid Receptors on Endothelin-1 (10−7 mol/L) Contractions of Mesenteric Resistance Arteries From C57BL/6 Mice: Comparison of Sham-Operated and Reduced Renal Mass Mice**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Contraction, %</th>
<th>By ANOVA, effects of:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SHAM</td>
<td>RRM</td>
</tr>
<tr>
<td>Vehicle</td>
<td>89.2±4.1</td>
<td>108.8±5.1</td>
</tr>
<tr>
<td>Tempol</td>
<td>79.0±3.4</td>
<td>73.6±2.5</td>
</tr>
<tr>
<td>MitoTEMPO</td>
<td>92.2±6.7</td>
<td>85.1±1.5</td>
</tr>
<tr>
<td>SC-560</td>
<td>84.1±5.4</td>
<td>96.1±2.3</td>
</tr>
<tr>
<td>Paracoxib</td>
<td>79.1±3.3</td>
<td>88.2±3.5</td>
</tr>
<tr>
<td>SC-560+Paracoxib</td>
<td>83.1±5.1</td>
<td>55.6±7.8</td>
</tr>
<tr>
<td>OKY-046NA</td>
<td>85.1±4.3</td>
<td>94.4±3.4</td>
</tr>
<tr>
<td>SQ-29,548</td>
<td>87.8±3.2</td>
<td>85.3±4.6</td>
</tr>
</tbody>
</table>

Mean±SEM values (n=6 per group). NS indicates nonsignificant; ROS, reactive oxygen species; and RRM, reduced renal mass.

**Figure 2.** Contractile concentration responses to endothelin-1 in mesenteric resistance arterioles from mice with reduced renal mass after bath addition of reactive oxygen species inhibitors (A): vehicle, tempol (10−4 mol/L) or mitoTEMPO (10−4 mol/L); or cyclooxygenase (COX) inhibitors (B): SC-560 (10−4 mol/L; COX-1 inhibitor), paracoxib (10−5 mol/L; COX-2 inhibitor), or SC-560 plus paracoxib; or thromboxane inhibitors (C): OKY-046NA (10−5 mol/L; thromboxane A2 synthase inhibitor) or SQ-29,548 (10−6 mol/L; thromboxane prostanoid inhibitor). Compared with vehicle: *P<0.05; **P<0.01; ***P<0.005.
Table 5. Basal Parameters, Plasma Endothelin-1, Renal Excretions of Makers of Reactive Oxygen Species, Thromboxane and Microalbumin, Vessel Structure, Maximum Vascular Contractions, and Reactive Oxygen Species Generation in Mesenteric Resistance Arterioles: Effects of Reduced Renal Mice and Thromboxane–Prostanoid Receptor Knockout

<table>
<thead>
<tr>
<th>Parameter</th>
<th>SHAM RRM</th>
<th>SHAM RRM</th>
<th>RRM Genotype Interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (BWt), g</td>
<td>24.9±0.5</td>
<td>30.5±1.6</td>
<td>NS NS NS</td>
</tr>
<tr>
<td>Kidney weight/BWt, mg/g</td>
<td>6.1±0.3</td>
<td>9.4±0.5‡</td>
<td>&lt;0.05 NS NS</td>
</tr>
<tr>
<td>Heart weight/BWt, mg/g</td>
<td>4.7±0.1</td>
<td>5.2±0.2</td>
<td>NS NS NS</td>
</tr>
<tr>
<td>Aorta weight/BWt, mg/g</td>
<td>0.14±0.005</td>
<td>0.15±0.008</td>
<td>NS NS NS</td>
</tr>
<tr>
<td>Plasma endothelin-1, pg/mL</td>
<td>2.2±0.3</td>
<td>4.6±0.2‡</td>
<td>&lt;0.05 NS NS</td>
</tr>
<tr>
<td>Urinary 8-isoprostane F2α/creatinine, ng/mg</td>
<td>1.43±0.18</td>
<td>5.36±0.62‡</td>
<td>&lt;0.05 NS NS</td>
</tr>
<tr>
<td>Urinary malondialdehyde/creatinine, μg/mg</td>
<td>47.7±9.1</td>
<td>72.5±7.5*</td>
<td>&lt;0.01 NS NS</td>
</tr>
<tr>
<td>Vessel media:lumen, μm2</td>
<td>0.13±0.01</td>
<td>0.23±0.02†</td>
<td>&lt;0.05 NS NS</td>
</tr>
<tr>
<td>Urinary TxB2/creatinine, ng/mg</td>
<td>1.2±0.1</td>
<td>2.7±0.2*</td>
<td>&lt;0.01 &lt;0.05 NS</td>
</tr>
<tr>
<td>Urinary microalbumin/creatinine, μg/mg</td>
<td>0.1±0.1</td>
<td>2.7±0.2*</td>
<td>&lt;0.01 &lt;0.05 NS</td>
</tr>
<tr>
<td>Vessel lumen CSA (×1000 μm2)</td>
<td>89.6±4.2</td>
<td>71.4±4.1</td>
<td>NS NS NS</td>
</tr>
<tr>
<td>Vessel media CSA (×1000 μm2)</td>
<td>12.0±1.2</td>
<td>16.4±2.5</td>
<td>NS NS NS</td>
</tr>
<tr>
<td>PE, 10−6 mol/L, contraction, %</td>
<td>76.4±5.9</td>
<td>54.4±7.3*</td>
<td>&lt;0.05 NS NS</td>
</tr>
<tr>
<td>ET-1, 10−6 mol/L, contraction, %</td>
<td>94.3±4.5</td>
<td>110.1±3.2*</td>
<td>&lt;0.01 &lt;0.001 NS</td>
</tr>
<tr>
<td>EtOH/DE, ∆f0</td>
<td>81.3±4.5</td>
<td>106.7±2.3*</td>
<td>&lt;0.01 &lt;0.05 NS</td>
</tr>
</tbody>
</table>

Mean±SEM values (n=6 per group). CSA indicates cross-sectional area; ET-1, endothelin-1; NS, nonsignificant; PE, phenylephrine; ROS, reactive oxygen species; RRM, reduced renal mass; TP-R, thromboxane prostanoid receptors; and TxB, thromboxane B.

Contraction and ROS Generation With ET-1 in Mesenteric Resistance Arterioles

Vessels from mice with RRM had reduced contractions to PE, but increased contractions to U-46,619 and ET-1 and generated more cellular and mitochondrial ROS with 10−7 mol/L of ET-1 than SHAM mice (Figure 1 and Table 3).

Effect of Metabolism of Cellular or Mitochondrial ROS or Blockade of COXs, TxA2-S, or TP-Rs on Contractions to ET-1 in Mesenteric Resistance Arterioles

The enhanced contractions to ET-1 in vessels from mice with RRM were reduced by incubation with tempol, MitoTEMPO, or paracoxib (a selective COX-2 inhibitor) alone or plus SC-560 (a selective COX-1 inhibitor) or SQ-29,548 (a TP-R inhibitor) but not with SC-560 alone or OKY-046NA (a TxA2-S inhibitor; Table 4; Figure 2).

Effect of TP-Rs in Mice With RRM

Similar to C57/B6 mice of the prior series, TP+/+ mice with RRM had increased plasma ET-1 and increased excretion of 8-Iso, MDA, TxB2, and microalbumin (Table 5). Their mesenteric resistance arterioles had increased vascular remodeling, decreased contractions to PE, but increased contractions to U-46,619 and ET-1 (Figure 3) and increased cellular ROS and mitochondrial ROS with ET-1 (Figure 4). TP-R−/− abolished all of these effects of RRM, except for the reduced PE contractions.

Discussion

We confirm that 3 months of RRM in C57Bl/6 mice increases oxidative stress, albuminuria, and growth of the remaining renal mass. The new findings are that these mice had increased plasma ET-1 and generated more TxA2, their mesenteric resistance arterioles were considerably remodeled and had reduced contractions to PE, but enhanced contractions to U-46,619 and ET-1 and enhanced cellular and mitochondrial ROS with ET-1. The enhanced ET-1 contractions were dependent on cellular and mitochondrial ROS and on TP-R and products of COX-2. Unlike TP-R+/+, TP-R+/− mice with RRM did not have significantly enhanced plasma levels of ET-1 or enhanced contractions or cellular or mitochondrial ROS in response to ET-1 and did not have vascular remodeling. Thus, TP-Rs mediate enhanced generation of ET-1 and microvascular ROS and enhanced contractility and remodeling in this mouse model of CKD. Figure 5 presents a schema for the proposed central role for TP-Rs in progressive CKD.

TP-Rs are activated by PGH2 (a primary product of COX-1 and -2), TxA2, and by the stable mimetic, U-46,619. The vascular protein expression of both COX-2 and TP-Rs was upregulated in mice with RRM, and the enhanced responsiveness to ET-1 was dependent on COX-2 and TP-Rs (although blockade of COX-1...
with COX-2 was more effective than COX-2 alone, suggesting some adaptive interaction). Because blockade of TxA₂S did not prevent enhanced ET-1 contractions, it is likely that PGH₂, generated by COX-2>COX-1, was the principal PG activating the TP-Rs. Activation of the TP-Rs enhances the generation of PGs and TxA₂, which further activate the TP-R in a feed-forward manner.³²,³³ This may account for the reduced excretion of TxB₂ in TP-R⁻/⁻ mice with RRM. Although mice with RRM had increased TP-R protein expression and increased TP-R responses to U-46,619, TP-R mRNA was unchanged. This may relate to oxidative stress that post-transcriptionally prevents TP-R protein degradation and enhances its membrane expression.³⁴

The enhanced contractions to ET-1 and U-46,619 in vessels from mice with RRM were probably not a consequence of vascular remodeling because contractions to PE were actually diminished. Adrenergic agonists usually do not provoke vascular oxidative stress.³⁵ Moreover, the structural and functional changes in mice with RRM were likely independent of BP and uremia because prior telemetric measurement of mean arterial pressure were unchanged after 3 months of RRM, and the global glomerular filtration rate was reduced by only 33%.³³

ET-1 is generated in vascular endothelial cells. Its production is increased by ROS,³⁶ and CKD.¹⁹ Although ANG II increases ROS in vascular smooth muscle cells largely by activation of NADPH oxidase,³⁵ ET-1 also activates other source of ROS, including the mitochondria.³⁷,³⁸ The present study demonstrates that ET-1 activated ethidium:dihydroethidium fluorescence ratio and mitoSOX™ Red fluorescence,³⁹ and both tempol (distributed throughout the cell)⁴⁰,⁴¹ and mitoTEMPO

Figure 3. Effect of thromboxane prostanoid receptors and reduced renal mass on contractile responses in mesenteric resistance arterioles. Data from SHAM (open symbols) or reduced renal mass (RRM; shaded symbols) in thromboxane prostanoid receptors (TP-R)+/+ (circles) or TP-R⁻/⁻ (square) mice. Mean±SEM values (n=6 per group). Compared with TP-R+/+: *P<0.05; ***P<0.005.

Figure 4. Effect of thromboxane prostanoid receptors and reduced renal mass on cellular and mitochondrial reactive oxygen species generation with 10⁻⁷ mol/L endothelin-1 in mesenteric resistance arterioles. Means±SEM values (n=6 per group) for cellular reactive oxygen species (ROS) generation (A) and mitochondrial ROS generation (B) from thromboxane prostanoid receptors (TP-R)+/+ or TP-R⁻/⁻ mice from SHAM (open boxes) or reduced renal mass (RRM; shaded boxes) groups. Compared with TP-R+/+: *P<0.05.
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(prepartitioned into mitochondria) prevented the enhanced contractility to ET-1 in vessels from mice with RRM. Thus, both sources of ROS were activated in mice with RRM and apparently both contributed to enhanced contractions to ET-1. p22phox is an essential chaperone protein for neutrophil oxidases. It was strongly upregulated in vessels from mice with RRM and could account for the observed increase in cellular ROS because in vivo silencing of p22phox prevents the progressive increase in excretion of 8-Iso and the hypertension of rats infused with ANG II. Neutrophil oxidases-2 is expressed in resistance arterioles and the kidney and was upregulated >3-fold in the kidneys of mice with RRM. An uncoupled endothelial NOS may have contributed also to the increased ROS.

In contrast to TP-R+/+, TP-R−− mice with RRM did not have significantly increased plasma levels of ET-1 or excretions of MDA, TxB2, or microalbumin or significantly enhanced cellular or mitochondrial ROS or contractions to ET-1 (although there were trends suggesting some residual effects). However, the reduced contractions to PE persisted in TP-R−− mice with RRM, which might represent downregulation of vascular α-adrenoreceptors during enhanced sympathetic nervous activity in RRM. The absence of vascular remodeling in arterioles from TP-R−− mice with RRM may represent less vascular ROS. Vascular remodeling in mice with ANG II is dependent on TP-Rs in vascular smooth muscle cells. Our findings extend microvascular studies that have reported that ROS enhance TP-R activity and responsiveness to ANG II and ET-1 by demonstrating that this pattern occurs in a model of CKD and that vascular TP-Rs are required to generate cellular and mitochondrial ROS with ET-1. Thus, TP-Rs are both upstream and downstream of ROS and thereby may play essential mediating and reinforcing roles in the generation of ROS from cellular and mitochondrial sources. They could thereby enhance remodeling and contractility of microvessels in CKD.

**Figure 5.** A flow diagram to demonstrate the potential central role for thromboxane prostanoid receptors (TP-R) in mediating the increased endothelin-1 (ET-1), reactive oxygen species (ROS), and microvascular remodeling that may contribute to progression of chronic kidney disease (CKD). RRM indicates reduced renal mass.
Perspective
Future CVD events are predicated by endothelial dysfunction and vascular remodeling, which are frequently accompanied by oxidative stress, as in CKD. ROS, ET-1, and TxA₂ are all increased in patients with CKD. The vascular remodeling, enhanced ROS, and responsiveness to ET-1 and thromboxane in mice with RRM were prevented by genetic deletion of TP-Rs. Thus, TP-R antagonists, which have already been used in clinical trials, could be novel drugs to prevent vascular oxidative stress and CVD in patients with CKD.

ETA-R blockade improves renal function and reduces glomerulosclerosis in a rat model of RRM and markedly reduces albuminuria in patients with diabetic and nondiabetic CKD. Because we now show the importance of TP-Rs in activating the ET-1 system in RRM, TP-R antagonists may reduce renal disease progression in addition to vascular injury.

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Disclosures
None.

References


**Novelty and Significance**

**What Is New?**

- The plasma levels of endothelin-1 (ET-1), the microvascular protein expression of p22phox, cytochrome c oxidase-2, thromboxane prostanoid receptors (TP-Rs), and endothelin-A receptors, the remodeling and the contractions to both ET-1 and thromboxone are increased in a mouse model of chronic kidney disease.
- The increases in microvascular cellular and mitochondrial reactive oxygen species of mice with reduced renal mass depend on TP-Rs.
- TP-R gene deletion prevents ET-1 generation, microvascular remodeling, enhanced contractile response, and reactive oxygen species generation in mice with reduced renal mass.

**What Is Relevant?**

- The results from clinical trials in patients treated with TP-R antagonists are promising. Vascular TP-Rs are novel targets to correct the remodeling, dysfunction, and oxidative stress in microvessels that precedes cardiovascular disease in patients with chronic kidney disease.

**Summary**

Mice with reduced renal mass have increased ET-1 and microvascular remodeling and enhanced ET-1-induced cellular and mitochondrial reactive oxygen species and contractions that are mediated by cytochrome c-2 products activating TP-Rs. Thus, TP-Rs can be upstream from reactive oxygen species and contribute to vascular dysfunction in chronic kidney disease.
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Thromboxane prostanoid receptors enhance contractions and oxidative stress in microvessels from mice with CKD

Cheng Wang1,2, Zaiming Luo1, Donald Kohan4, Anton Wellstein2, Pedro A. Jose5, William J. Welch1, Christopher S. Wilcox1, and Dan Wang1

1Hypertension, Kidney and Vascular Research Center and Division of Nephrology and Hypertension and 3Lombardi Cancer Center, Georgetown University, Washington, DC;
2Department of Nephrology, The Third Hospital at Sun Yat-sen University, Guangzhou, P.R.China ; 4Division of Nephrology, University of Utah and 5Division of Nephrology, Department of Medicine and Department of Physiology, University of Maryland, Baltimore, MD, USA

Address for Correspondence:

Dan Wang, M.D., Ph.D.
Division of Nephrology and Hypertension
Georgetown University Medical Center
Building D, Room 399, 4000 Reservoir Road NW
Washington, DC 20007
Phone: 202-687-0887

Fax: 202-687-4194

Email: dw32@georgetown.edu

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Methods

Measurement of urinary 8-Iso, TxB2, MDA, and microalbumin

Urine samples were collected from mice housed for 24 hours in metabolic cages. For 8-Iso F2α and TxB2, the urine was purified by absorption onto bond elut phenylboronic acid solid phase extraction cartridges (Agilent Technologies, Columbia, MD). Samples were diluted 1:1 with EIA buffer (Cayman Chemical, Ann Arbor, MI) to yield 1.0ml volume, [3H]-prostaglandin E2 added to measure recovery and diluted to 10 l. These samples were processed through the cartridges under minimum vacuum pressure, and eluted with 1.0 ml methanolic acetic acid. The eluent was evaporated under a steam of nitrogen and redissolved in EIA buffer. Levels were measured by commercially available enzyme-linked assay kits (Cell Biolabs, Inc, San Diego, CA; QAYEE-BIO, Shanghai, China).

Urinary MDA was measured by a TBARs assay kit (Cayman Chemical Company, Ann Arbor, MI), microalbumin by a murine microalbuminuria ELISA kit (Exocell, Philadelphia, PA), and creatinine by creatinine chemical assay kit (Exocell, Philadelphia, PA). All parameters were normalized with urinary creatinine levels.

Protein expression of p22phox, p47phox, COX-1, COX-2, TP-R and ETA-R in mesenteric resistance arterioles:

Vessels from SHAM and RRM mice were dissected under a microscope and stored at -80 ºC. Mesenteric resistance arterioles were lysed with RIPA buffer (Sigma, Cat: R0278, St. MO), containing a cocktail of protease inhibitors (Roche Diagnostics, Indianapolis, IN) added at 4ºC and homogenized in lysing matrix D (MP biomedicals, Solon, OH, USA). The cell lysates were centrifuged at 10,000 g for 10 minutes at 4ºC. Supernatant protein concentration was measured (Bio-Rad, protein assay kit, Cat: 500-0006, Hercules, CA) and adjusted to a final loading protein concentration of 1.5 µg·µl⁻¹. An initial gel was stained with Coomassie blue to confirm equal loading. Immunoblotting was performed by 10% SDS-PAGE (Criterion Ready Gels, Bio-Rad, Hercules, CA) and 20-40 µg of protein were electrophoretically transferred from the gel onto polyvinylidene difluoride membranes.

After 60 min of incubation in 5% milk, the membranes were probed overnight at 4ºC with different primary antibodies for the proteins: ETA-R (1:1000 dilution, Thermo Scientific, Rockford IL); p22phox (1:200 dilution, Santa Cruz, Dallas, TA); p47phox (1:1000 dilution, Sigma Aldrich, St Louis, MO), Cox-1(1:1000 dilution, Cell signaling Technology, Danvers, MA); COX-2 (1:1000 dilution, Cell signaling Technology,
Danvers, MA); TP-receptor (1:1500 dilution, Thermo Scientific, Rockford IL). β-actin antibody was used (1:25000, Sigma-Aldrich, St. Louis, MO) as a control for equal loading of samples. The membranes were exposed to secondary antibodies, diluted to 1:5000 (goat anti-rabbit IgG conjugated with horseradish peroxidase, Pierce no. 31463,) for 1 hour at room temperature. The specific bands were visualized and analyzed densitometrically with a Fluorochem Enhanced Chemiluminescence system (FluorChem E system, ProteinSimple, Santa Clara, CA).

**RNA isolation from mesenteric resistance arterioles and real-time quantitative RT-PCR:**

Total RNA isolation and real-time quantitative PCR was performed as previously described with some modifications. Briefly, RNA was isolated from mouse frozen mesenteric resistance arterioles with miRNeasy Mini kit and treated with Rnase-Free DNase set (Qiagen, Valencia, CA) following the manufacturer’s instruction manual. Reverse transcription of RNA was performed with a High Capacity RNA- to- cDNA kit (Applied Biosystems, Foster City, CA). Primers and probes for mouse p22phox (ID: Mm00514478_m1), endothelin receptor type-A (ID: Mm01243722_m1), preproendothelin-1 (ID: Mm00438656_m1), COX-1 (ID: Mm00477214_m1), COX-2 (ID: Mm00478374_m1), thromboxane-A2 receptor (ID: Mm00436917_m1), and were purchased from Applied Biosystems for gene expression assays. Real time PCR was carried out in a StepOne Plus Real-time PCR system (Applied Biosystems). The comparative [DELTA][DELTA]CT method was used for relative quantification and statistical analysis.

Primers and probes for mouse p22phox, ETA-R, preproendothelin-1, COX-1, COX-2 and TP-R were purchased from Applied Biosystems and real time PCR was carried out in a StepOne Plus Real-time PCR system (Applied Biosystems). The comparative [DELTA][DELTA]CT method was used for relative quantification and statistical analysis.
Preparation and study of mesenteric resistance arterioles: Four segments of second-generation branches of mesenteric arteries (about 2 mm in length with an internal diameter of less than 170 μm) were mounted on two 40 μm stainless steel wires in an isometric four-chamber Mulvany-Halpern myograph (Model M610, Darush Myo Technology A/S, Aarhus, Denmark). One wire was attached to a force transducer and the other to a micrometer to enable the wall tension to be measured at a predetermined internal circumference. Both the dissection and mounting of the vessels were carried out in cold (4°C) physiological salt solution (PSS).

Experimental protocol for mesenteric resistance arterioles: Vessels were equilibrated for 30 min in PSS (in mmol·L⁻¹) (NaCl 118, NaCO₃ 25, KCl 4.5, CaCl₂ 2.5, MgSO₄ 1.0 and glucose 6.0) at pH 7.4 and 37°C, bubbled with 5% CO₂ and 21% O₂. After equilibration, vessels were stimulated by a high-potassium (30 mmol·l⁻¹ KCl) physiological salt solution (KPSS) containing 10⁻⁵ mol·L⁻¹ norepinephrine (NAK). Contraction responses to NAK were defined as a 100% standard contraction. Concentration-dependent responses to phenylephrine (PE; 10⁻⁸ to 10⁻⁵ mol·L⁻¹), U-46,619 (10⁻⁹ to 10⁻⁶ mol·L⁻¹) or endothelin-1 (ET-1; 10⁻¹⁰ to 10⁻⁷ mol·L⁻¹) were assessed by cumulative addition to the organ bath. A fluorescence microscope (Olympus IX 71, Olympus, Virginia, USA) equipped with RatioMaster™ spectrofluorometer system (Photo technology International, Canada) was used to evaluate ROS generation, as described previously and below.

Cellular and Mitochondrial ROS of mesenteric resistance arterioles

Dihydroethidium (DHE, Invitrogen, Carlsbad, CA) is a cellular ROS indicator that freely permeates into cells. It is oxidized by ROS into the highly fluorescent ethidium (Eth) which is trapped intracellularly and intercalated into DNA. The conversion of DHE to Eth is quantified by a ratiometric method using a dual wavelength determination using an excitation wavelength of 380 nm and an emission wavelength of 460 nm for DHE and an excitation wavelength of 480 nm and an emission wavelength of 605 nm for Eth.

The mitochondrial ROS indicator, mitoSOX red (Invitrogen, Carlsbad, CA) is a cell permeant derivative of hydroethidine. It rapidly and selectively targets mitochondria with positively charged triphenylphosphonium. Once in the mitochondria, the reagent is intercalated into the mitochondrial DNA and oxidized by O₂⁻, and exhibits red fluorescence (excitation/emission at λ = 510 nm/580 nm).

Mesenteric resistance arterioles were loaded with Hanks’ Balanced Salt Solution (HBSS) containing DHE (10⁻⁵ mol·L⁻¹) or MitoSOX Red (5x10⁻⁶ mol·L⁻¹, Invitrogen, Carlsbad, CA). After 30 min of equilibration, 10⁻⁷ mol·L⁻¹ of endothelin-1 or vehicle were added and the Eth:DHE ratio were assessed at 1 min periods. The changes in ROS after
minutes of incubation were monitored with a real time photomultiplier that recorded either the ratio of Eth:DHE or mitoSOX fluorescence\(^6,7\) (Felix32, Photon Technology International, Lawrenceville, NJ). The system utilized an Olympus IX70 fluorescence microscope with ratiomaster fluorescence measurements with a dual photomultiplier system (Photon Technology International, Lawrenceville, NJ). Fluorescent images were captured using an Olympus (Melville, NY) IX71 fluorescent microscope at 20× magnification equipped with a HQ2 CCD cooled camera and further analyzed by Nikon element advance research microscope imaging software.
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Figure S1. Individual Western blots from mesenteric resistant arteriolar lysate (Panel A) and group mean ± SEM densitometry values for endothelin type A receptor protein expression in mesenteric resistance arteriols (Panel B). SIAM, open box and reduced renal mass, solid box. Comparing groups: *, P<0.05
Figure S2. Individual Western blots from mesenteric resistance arteriolar lysates (Panel A) and group mean ± SEM densitometry values for the p47phox (Panel B) and p22phox (Panel C) components of nicotinamide adenine dinucleotide phosphate oxidase. SHAM, open boxes and reduced renal mass, shaded boxes. Comparing groups: **, P<0.01
Figure S3. Individual Western blots from mesenteric resistance arteriolar lysates (Panel A) and group mean ± SEM densitometry values for thromboxane-prostanoid receptor (Panel B), cyclooxygenase-1 (Panel C) and cyclooxygenase-2 (Panel D). SilAM, open boxes and reduced renal mass, solid boxes. Comparing groups: **, P<0.01.
Figure S4. Mean ± SEM values (n=4 to 6 per group) for mRNA expression in mesenteric resistance arterioles for endothelin type A receptor (Panel A), preproendothelin-1 (Panel B), p27Kip1 (Panel C), cyclooxygenase-1 (Panel D), cyclooxygenase-2 (Panel E) and thromboxane-prostanoid receptor (Panel F). SHAM, open boxes and reduced renal mass, solid boxes. Compared to SHAM: * P<0.05; ** P<0.01; *** P<0.005