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Selective COX-2 Inhibitors and Renal Injury in Salt-Sensitive Hypertension

Matthias Hermann, Sidney Shaw, Eva Kiss, Giovanni Camici, Nico Bühler, Remy Chenevard, Thomas F. Lüscher, Hermann J. Gröne, Frank Ruschitzka

Abstract—In view of the ongoing controversy of cardiorenal safety of selective COX-2 inhibitors (coxibs), the present study was designed to examine the effects of 2 different coxibs, celecoxib and rofecoxib, compared with a traditional NSAID, diclofenac, and placebo on renal morphology and function in salt-sensitive hypertension. Salt-sensitive (DS) and salt-resistant (DR) Dahl rats were fed with NaCl-enriched diet (4% NaCl) for 8 weeks.Diclofenac (DS-diclofenac), rofecoxib (DS-rofecoxib), celecoxib (DS-celecoxib), or placebo was added to chow from weeks 6 to 8. Immunostaining for monocytes/macrophages (ED1) and cytotoxic T lymphocytes (CD8) was performed. In addition, renal morphology and proteinuria were assessed. Renal cortex mRNA was isolated for determination of COX-2, eNOS, and CRP mRNA by real-time reverse-transcriptase polymerase chain reaction. Untreated hypertensive animals showed glomerular injury including collapsing glomerulopathy, mesangial sclerosis, extracapillary proliferation, protein drops, and an especially high grade of glomerulosclerosis (P<0.05 versus DR-placebo) and CD8-positive and ED1-positive cells (P<0.01 versus DR-placebo), which was improved by celecoxib but not by diclofenac and rofecoxib. C-reactive protein mRNA in renal cortex was increased in DS-placebo animals (P<0.05 versus DR-placebo) and normalized by celecoxib (P<0.05 versus DS-placebo), whereas eNOS mRNA was decreased in the DS-rofecoxib group (P<0.05 versus DR-placebo, DS-celecoxib, and DS-diclofenac). Proteinuria was observed in hypertensive animals (P<0.0001 versus DR-placebo), increased by rofecoxib (P<0.05 versus DS-placebo), and normalized by celecoxib (P=0.0015 versus DS-placebo). This head-to-head comparison of selective and nonselective COX inhibitors demonstrates differential effects of coxibs on renal morphology and function in salt-dependent hypertension. (Hypertension. 2005; 45:193-197.)

Key Words: hypertension ■ kidney ■ proteinuria

Both cyclooxygenases (COX-1 and COX-2) are expressed in the human kidney, and COX-derived prostaglandins play a pivotal role in the maintenance of renal blood flow and glomerular filtration. COX-1 is found in the glomerulus and afferent arteriole, whereas COX-2 is expressed in the podocytes, thick ascending limb of the loop of Henle, macula densa, and afferent arteriole. Upregulation of the COX-1 and/or the COX-2 isoforms has been described in several clinical and experimental conditions characterized by inflammation, suggesting that activation of COX-2 might play an important role in the pathogenesis and progression of nephropathies.

There is increasing evidence of differential clinical effects of selective COX-2 inhibitors. Therefore, the present study was designed to examine the effect of celecoxib, rofecoxib, and diclofenac, a nonselective nonsteroidal anti-inflammatory drug with a strong relative COX-2/COX-1 selectivity, on the development of progressive renal dysfunction in an animal model of salt-dependent hypertension that is known to develop severe renal damage during high-salt diet.

Methods

Animals and Experimental Groups

The present article is a follow-up study of a previously published article now focusing on renal injury in the same animals and experimental protocol. In brief, male Dahl rats (salt-sensitive [DS] and salt-resistant [DR]; 11 weeks old, n=6 to 8 per group obtained from M&B; Ry, Denmark) were fed 4% NaCl-enriched chow for 8 weeks. From week 6 to week 8, diclofenac (6 mg/kg per day), rofecoxib (2 mg/kg per day), celecoxib (25 mg/kg per day), or placebo were added to chow. Food and drug intake was calculated every day. All rats were kept under a 12-hour light/12-hour dark cycle. Systolic arterial blood pressure and heart rate were measured by the tail-cuff method with a pulse transducer (model LE 5000; Letica) at weeks 1, 5, and 8. Study design and experimental protocols were approved by the institutional animal care committee (Kommiss-
sion für Tierversuche des Kantons Zürich, Switzerland) and were in strict accordance with our institutional guidelines and with international standards for animal care.

**Tissue Harvesting**
Animals were anesthetized with pentobarbital (50 mg/kg IP) after 8 weeks. Kidneys were handled as previously described. Functional Renal Parameters
Proteinuria, creatinine clearance, and fractional sodium excretion were assessed by the use of metabolic cages for 24 hours after a 24-hour run-in phase before harvesting.

**Morphology and Immunohistochemistry**
Light microscopy was performed on 3-μm sections stained by periodic acid-Schiff as recently described. Immunohistochemistry was performed for monocytes/macrophages (ED1) and cytotoxic T lymphocytes (CD8).

**Immunoblotting and Cytokine Assay**
Western blotting was performed with appropriate antibodies and normalized to α-tubulin.

**Differences Among Groups**

<table>
<thead>
<tr>
<th>Parameter, Unit</th>
<th>DR-Placebo</th>
<th>DS-Placebo</th>
<th>DS-Celecoxib</th>
<th>DS-Diclofenac</th>
<th>DS-Rofecoxib</th>
</tr>
</thead>
<tbody>
<tr>
<td>Physiological and biochemical characteristics</td>
<td></td>
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<tr>
<td>Systolic blood pressure, mm Hg</td>
<td></td>
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<tr>
<td>Week 1</td>
<td>162.3 ± 3.5*</td>
<td>181.1 ± 5.6</td>
<td>179.1 ± 2.4</td>
<td>176.7 ± 5.4</td>
<td>182.8 ± 6.7</td>
</tr>
<tr>
<td>Week 5</td>
<td>161.1 ± 1.1*</td>
<td>203.8 ± 1.3</td>
<td>202.1 ± 0.5</td>
<td>204.8 ± 1.0</td>
<td>204.7 ± 1.2</td>
</tr>
<tr>
<td>Week 8</td>
<td>158.2 ± 4.5*</td>
<td>222.4 ± 2.9</td>
<td>213.2 ± 2†</td>
<td>225.5 ± 2‡</td>
<td>225.4 ± 1§</td>
</tr>
<tr>
<td>Heart rate, beats/min</td>
<td>278 ± 12.3</td>
<td>283 ± 9.8</td>
<td>279 ± 14.7</td>
<td>273 ± 15.4</td>
<td>283 ± 13.9</td>
</tr>
<tr>
<td>Body weight, g</td>
<td>393 ± 6.5*</td>
<td>433 ± 5.7</td>
<td>440 ± 4.1</td>
<td>441 ± 2.4</td>
<td>472 ± 5.2‡</td>
</tr>
<tr>
<td>Proteinuria, mg/d</td>
<td>37.0 ± 4.6§</td>
<td>302.8 ± 109.9</td>
<td>86.1 ± 75.1§</td>
<td>289.9 ± 132</td>
<td>398.7 ± 78.3</td>
</tr>
<tr>
<td>FE Na, %</td>
<td>2.4 ± 0.5</td>
<td>3.3 ± 1.2</td>
<td>1.4 ± 1.4§</td>
<td>3.1 ± 0.8</td>
<td>3.2 ± 1.3</td>
</tr>
<tr>
<td>Sodium plasma, mmol/L</td>
<td>137 ± 3.5</td>
<td>136 ± 2.8</td>
<td>135 ± 2.3</td>
<td>136 ± 3.9</td>
<td>136 ± 1.0</td>
</tr>
<tr>
<td>Urine volume, mL/d</td>
<td>47.5 ± 8.0§</td>
<td>69.5 ± 12.9</td>
<td>35.6 ± 20.2§</td>
<td>66.6 ± 10.4</td>
<td>76.1 ± 15.8</td>
</tr>
<tr>
<td>Creatinine plasma, μmol/L</td>
<td>46.25 ± 3.05§</td>
<td>62.50 ± 4.19</td>
<td>55.20 ± 3.71</td>
<td>53.00 ± 1.93</td>
<td>63.38 ± 5.20</td>
</tr>
<tr>
<td>Morphology of renal vessels</td>
<td></td>
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</tr>
<tr>
<td>Endothelial activation/proliferation, %</td>
<td>5.60 ± 1.95§</td>
<td>30.41 ± 9.24</td>
<td>10.33 ± 6.58†</td>
<td>47.30 ± 10.29</td>
<td>52.86 ± 8.67</td>
</tr>
<tr>
<td>Broadening of adventitia, %</td>
<td>1.29 ± 0.87§</td>
<td>33.30 ± 8.29</td>
<td>6.78 ± 6.78§</td>
<td>51.33 ± 12.64</td>
<td>43.65 ± 10.43</td>
</tr>
<tr>
<td>SMC necrosis, %</td>
<td>0.00 ± 0.00</td>
<td>1.58 ± 1.03</td>
<td>1.23 ± 1.23</td>
<td>1.52 ± 0.96</td>
<td>7.03 ± 1.81‡</td>
</tr>
<tr>
<td>Subintimal insudation, %</td>
<td>0.63 ± 0.63††</td>
<td>9.16 ± 4.89</td>
<td>3.11 ± 1.32**</td>
<td>19.70 ± 9.18</td>
<td>22.58 ± 5.35</td>
</tr>
<tr>
<td>Immunohistochemistry and morphology of glomeruli</td>
<td></td>
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</tr>
<tr>
<td>ED-1, cells/glom</td>
<td>0.538 ± 0.042§</td>
<td>3.080 ± 1.082</td>
<td>1.033 ± 0.400§</td>
<td>3.825 ± 0.557</td>
<td>5.122 ± 0.69</td>
</tr>
<tr>
<td>CD8, cells/glom</td>
<td>0.313 ± 0.035§</td>
<td>1.280 ± 0.343</td>
<td>0.550 ± 0.109§</td>
<td>1.625 ± 0.368</td>
<td>1.538 ± 0.194</td>
</tr>
<tr>
<td>Sclerosis index</td>
<td>0.54 ± 0.31†</td>
<td>43.70 ± 16.29</td>
<td>19.33 ± 9.18**</td>
<td>34.98 ± 8.46</td>
<td>54.85 ± 11.99</td>
</tr>
<tr>
<td>Mesangial sclerosis, %</td>
<td>0.68 ± 0.36§</td>
<td>15.31 ± 2.47</td>
<td>5.78 ± 1.86§</td>
<td>13.22 ± 3.92</td>
<td>11.78 ± 1.78</td>
</tr>
<tr>
<td>Extracapillary proliferation, %</td>
<td>0.00 ± 0.00§</td>
<td>2.15 ± 0.56</td>
<td>0.67 ± 0.36</td>
<td>2.78 ± 0.97</td>
<td>3.61 ± 0.97</td>
</tr>
<tr>
<td>Protein droplets, %</td>
<td>0.06 ± 0.06*</td>
<td>20.22 ± 1.92</td>
<td>20.32 ± 4.34</td>
<td>13.68 ± 4.39</td>
<td>29.81 ± 4.00</td>
</tr>
<tr>
<td>Collapsing glomerulopathy, %</td>
<td>0.15 ± 0.15§</td>
<td>1.24 ± 0.42</td>
<td>0.79 ± 0.46</td>
<td>1.20 ± 0.62</td>
<td>1.24 ± 0.29</td>
</tr>
</tbody>
</table>

FE Na indicates fractional excretion of sodium; SMC, smooth muscle cell.
Results are presented as mean ± SEM. Blood pressure results have been published previously.

P < 0.001 vs DS-placebo, DS-celecoxib, DS-diclofenac, DS-rofecoxib.
†P < 0.001 vs DR-placebo, DS-placebo, DS-diclofenac, DS-rofecoxib.
‡ P < 0.005 vs DS-placebo.
§P < 0.01 vs DS-placebo, DS-diclofenac, DS-rofecoxib.
¶P < 0.05 vs DS-placebo, DS-rofecoxib.
**P < 0.005 vs DS-placebo.
††P < 0.005 vs DS-diclofenac, DS-rofecoxib.

**Real-Time Quantitative Reverse-Transcriptase Polymerase Chain Reaction**
Real-time quantitative polymerase chain reaction was performed as previously described. Gene expression was normalized to the endogenous control, GAPDH mRNA, and the amount of target gene mRNA expression in each sample expressed relative to that in controls. Higher Δ cycle time (ΔCT) values express lower mRNA levels. The following primers and probes were used: COX-2: forward 5′ TGC TAC TTT TCC AGG ATG GAA A 3′; reverse 5′ GAG TGT CTT TGA CTG TGG GAG GAT G 3′; and probe 5′ FAM (TAMRA probe) 5′ TGA AAT ATC AGG (TCA) TCG GTG GAG AGG TG 3′; for rat C-reactive protein (CRP), forward: 5′ TAC TGC GTA TGT GTC CCT GGA A 3′, reverse 5′ ACA TCA GCC TGG GCA TAG AGA 3′, probe 5′ TCA AAG CCA CTA CAG CAA GCC TTC ACT G 3′; and for eNOS, forward: 5′ CTA CCG GGA CGA GGT ACT GG 3′, reverse 5′ GGA AAA GCC GGT GAG GAC TT 3′, probe 5′ CGC CCA GCA GCC TGG AGT GTT T 3′.

**Calculations and Statistical Analysis**
Data are given as mean ± SEM. For multiple comparisons, results were analyzed by ANOVA, followed by Mann–Whitney or t test when appropriate. A value of P < 0.05 was considered significant.
Results

Hemodynamics and Vital Parameters
The blood pressure results were published recently in our previous publication focusing on endothelial function. Blood pressure increased in all DS animals, as published recently. Blood pressure further increased in the drug-treated, as well as in the placebo-treated, animals from week 5 to week 8 (Table). It is of note that blood pressure levels did not differ from placebo in the respective groups after 5 weeks (Table), the time point when all animals were randomized to treatment with the respective study drug or placebo. Importantly, the blood pressure increase in DS animals was more pronounced with rofecoxib and diclofenac treatment, but attenuated in the celecoxib treated animals. Body weight increased in all hypertensive rats and was more pronounced in the rofecoxib group (Table). Chow intake was measured daily and was comparable in all hypertensive groups.

Renal Function
Proteinuria was evident in all hypertensive animals. Proteinuria remained unchanged during treatment with diclofenac, was further increased by rofecoxib, but was reduced by celecoxib (Table).

Renal Vessel Morphology
Untreated hypertensive animals showed endothelial activation/proliferation, subintimal plasma insudation, smooth muscle necrosis of the vessel wall, and broadened adventitia, indicating injury of preglomerular vessels. These lesion formations were all reduced by celecoxib, but were increased by diclofenac and rofecoxib (Table, Figure 1).

Glomerular Morphology
The number of normal glomeruli and normal vessels was decreased in all hypertensive animals (versus normotensive DR-placebo). DS-placebo animals showed signs of glomerular injury, including collapsing glomerulopathy, mesangial sclerosis, mesangiolysis, extracapillary proliferation, protein decreases, and especially high grade of glomerulosclerosis when compared with normotensive animals. Treatment with celecoxib induced a significant improvement of these morphological changes, whereas a further worsening of the glomerular lesions in the diclofenac- and rofecoxib-treated animals was observed (P<0.05 versus DS-placebo for protein decreases; Table, Figure 1).

Immunohistochemistry
The number of monocytes/macrophages (ED-1-positive cells) was increased in hypertensive animals, further elevated in the rofecoxib-treated animals, but reduced by celecoxib (Figure 2). CD8-positive T lymphocytes were increased in hypertensive animals and reduced by celecoxib, only (Table).

CRP, eNOS, and COX-2 mRNA and COX-2 Protein Levels in Renal Cortex
CRP mRNA expression was increased in hypertensive animals as determined after normalization to GAPDH (15.99±1.08 ΔCT versus 19.06±1.02 ΔCT; P<0.05 versus DR-placebo).
Celecoxib normalized CRP mRNA levels in renal cortex (19.57±1.19 ΔCT; *P*<0.05 versus DS-placebo), whereas CRP mRNA levels remained unchanged in the rofecoxib-treated and diclofenac-treated animals (17.59±0.90 ΔCT and 16.99±1.14 ΔCT, respectively).

COX-2 mRNA expression was unaffected by salt-induced hypertension or any drug treatment. mRNA levels were 12.15±0.39 ΔCT in the DR-placebo, 11.92±0.35 ΔCT in the DS-placebo, 11.76±0.58 ΔCT in the DS-celecoxib, 11.04±0.31 ΔCT in the DS-diclofenac, and 11.52±0.27 ΔCT in the DS-rofecoxib group. COX-2 protein levels were comparable in all groups (DR-placebo 0.81±0.02, DS-placebo 1.04±0.03, DS-celecoxib 0.82±0.18, DS-diclofenac 0.83±0.26, DS-rofecoxib 0.91±0.33).

DS-placebo animals showed a trend toward decreased eNOS mRNA levels (8.35±0.23 ΔCT versus 7.84±0.24 ΔCT; *P*=0.093 versus DR-placebo). eNOS mRNA levels were decreased in the DS-rofecoxib group (8.59±0.15 ΔCT versus 7.84±0.24 ΔCT, 7.79±0.15 ΔCT, and 7.74±0.20 ΔCT; *P*<0.05 versus DR-placebo, DS-celecoxib, and DS-diclofenac).

**Discussion**

This head-to-head comparison of selective and nonselective COX inhibitors demonstrates differential effects of coxibs on renal function in salt-dependent hypertension. Celecoxib, but not rofecoxib or diclofenac, normalized proteinuria, decreased renal injury, and reduced cellular inflammation in this model of salt-sensitive hypertension.

Arterial hypertension is known to induce vascular, glomerular, and interstitial renal injury. In the present study, salt-induced hypertension was associated with signs of glomerular and vascular injury, as indicated by increased glomerulosclerosis index, mesangial sclerosis, extracapillary proliferation, endothelial activation/proliferation, and broadening of adventitia. In addition, glomeruli and renal vessels showed increased numbers of proinflammatory mononuclear cells and increased levels of CRP mRNA. Proteinuria, a hallmark of glomerular injury, developed in hypertensive animals and was prevented by celecoxib, only. Furthermore, celecoxib, but not rofecoxib and diclofenac, improved glomerular, interstitial, and renal vascular changes, prevented accumulation of macrophages and lymphocytes, and normalized CRP mRNA levels in the kidney.

Inflammation is crucially involved in promotion and aggravation of renal injury after arterial hypertension. In a long time seen as a marker of inflammation only, reduces NO bioavailability, thus leading to atherosclerotic lesion formation and progression of atherosclerosis. It is of note that expression of CRP, which is synthesized in the liver and the kidney, and quenches eNOS expression in cultured endothelial cells, is upregulated in untreated hypertensive animals and normalized by celecoxib treatment under the conditions of the present study.

An increasing body of evidence suggests that oxidative stress accounts in large parts for reduced NO bioavailability. The present study demonstrates that celecoxib reduces accumulation of macrophages, which on activation by inflammatory stimuli, release bioactive lipid peroxidation products, inflammatory mediators, and large quantities of reactive oxygen species. Rofecoxib-treated hypertensive animals, however, showed a trend toward progression of hypertensive renal injury. In addi-
tion, an increase of body weight, indicating enhanced fluid retention in the absence of corresponding changes of sodium excretion, occurred in the rofecoxib-treated animals, only. This is consistent with results of recently published clinical studies demonstrating increased edema formation and exacerbation of heart failure in users of rofecoxib, but not celecoxib. Further support for differential effects of rofecoxib and celecoxib comes from large-scale population-based cohort and case-control studies demonstrating that the odds of hypertension, congestive heart failure, and myocardial infarction were greater in patients using rofecoxib. It is of note that in these studies, cardiovascular risk was increased in patients using rofecoxib across a wide dose range, thus indicating that COX-2-independent effects rather than the duration of drug exposure and relative degree of selectivity attained may account for some of the apparent differences between the coxibs. In addition, further evidence for a distinct heterogeneity within this class of drugs was provided by preclinical studies showing that celecoxib inhibited proliferation, induced apoptosis in the G1- phase of the cell cycle, and, at subapoptotic concentrations, increased the expression of inhibitory proteins during the G1-phase of the cell cycle in human vascular endothelial cells. In contrast, rofecoxib showed no effect on cell proliferation and cell cycle progression, even at higher concentrations. Furthermore, Yang et al demonstrated that celecoxib blocks the injury-induced Akt/GSK signaling axis, leading to an increase in vascular smooth muscle cell apoptosis and an inhibition of vascular smooth muscle proliferation and neointimal hyperplasia after angioplasty.

It is of note that celecoxib as a sulfonamide is more extensively distributed into tissues than the sulfone rofecoxib, suggesting that additional effects unrelated to COX-2 inhibition at the tissue level, such as oxidation of membrane proteins, should be considered. Notwithstanding drugs were given with the chow, a constant drug intake was ensured, and TxB2 of celecoxib is reported within the range of 10 to 12 hours, we cannot rule out the possibility that there were periods during the day in which COX-2 was not inhibited. This, however, appears unlikely to have contributed to the improvement of renal function and morphology observed with celecoxib compared with placebo-treated hypertensive animals. If a lesser degree of COX-2 inhibition would account for the differential renal effects, one would have expected a less pronounced deterioration in the celecoxib-treated animals (when compared with rofecoxib), but not an improvement, particularly when compared with hypertensive animals not exposed to a COX-2 inhibitor or traditional nonsteroidal anti-inflammatory drug. Despite similar levels of TxB2, PGI2, PGE2, COX-2 gene, and protein expression in the renal cortex in the different treatment groups, only celecoxib treatment reduced renal inflammation and injury, as well as plasma levels of 8-isoprostane and IL-1β, as already demonstrated in our previous publication.

Perspectives
The results of the present study add to the increasing body of evidence of a distinct heterogeneity within this widely prescribed class of drugs by demonstrating differential effects on renal function in salt-dependent hypertension. Whether and to what extent this translates into clinically relevant differences, however, remains to be addressed in properly designed event trials.

References