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Effect of flosulide, a selective cyclooxygenase 2 inhibitor, on passive Heymann nephritis in the rat

Cornelia Blume, Gunhild Heise, Anja Mühlfeld, Dieter Bach, Karsten Schrör, Claus Dieter Gerhardz, Bernd Grabensee, and Peter Heering

Klinik für Nephrologie und Rheumatologie, Institut für Pharmakologie, Institut für Pathologie der Heinrich-Heine Universität Düsseldorf, Düsseldorf, Germany

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Background. Nonsteroidal anti-inflammatory drugs (NSAIDs) induce an inhibition of cyclooxygenase (COX), an enzyme that makes prostaglandins. Two isoforms of COX exist: COX-1 represents the constitutively expressed enzyme, whereas COX-2 is the inducible isoform. This study investigated the role of COX-2 in the inflammatory processes of the kidneys of rats with passive Heymann nephritis (PHN), and focused of the effect of a selective COX-2 inhibitor, flosulide. COX-2–selective inhibitors are thought to represent potent anti-inflammatory agents without major renal side effects.

Methods. PHN was induced by injecting heterologous Fx1A antiserum into female Wistar rats. Two treatment groups, each consisting of 12 rats with PHN, received either 3 or 9 mg of flosulide/kg body wt/day and were compared with untreated controls. After four weeks, the generation of thromboxane B_2 (TxB_2) and 6-keto-$PGF_{1\alpha}$ were determined in renal tissue and in urine. COX-2 protein expression was investigated by Western blotting using a selective antibody.

Results. Rats with PHN exhibited a marked proteinuria of 71 ± 8 mg/24 hr as compared with 2.0 ± 0.3 mg/24 hr in healthy controls ($P < 0.01$). Treatment with flosulide reduced the proteinuria to 26.1 ± 9 mg/24 hr at 3 mg flosulide/kg body wt/day and 35.5 ± 6 mg/24 hr at 9 mg/kg body wt/day, which was equivalent to a reduction of proteinuria by a maximum of 65% ($P < 0.05$). This was accompanied by an increase in glomerular TxB_2 from 3073 ± 355 to 5255 ± 1041 pg/mg glomerular protein and 6-keto-$PGF_{1\alpha}$ from 1702 ± 161 to 2724 ± 770 pg/mg glomerular protein in rats with PHN. COX-2 protein expression was also highly elevated in comparison to healthy controls. Low-dose flosulide treatment had no effect on COX protein expression and renal prostaglandin formation. High-dose flosulide treatment reduced renal prostaglandin production and caused a marked decline in COX-1 and COX-2 protein expression. Urine prostanoid excretion remained unchanged in all therapeutic groups. There was a small though significant reduction in renal creatinine clearance from 0.86 ml $\pm$ 0.2/min in untreated controls to 0.6 ml $\pm$ 0.1/min in flosulide-treated rats with PHN ($P < 0.01$) after four weeks.

Conclusions. Under the influence of flosulide, a highly COX-2–selective inhibitor, we observed an antiproteinuric drug effect. The inflammation in PHN induced COX-2 protein expression that was not affected by low-dose flosulide. COX-1 and COX-2 protein expression was affected by high-dose flosulide, which therefore might lose its selectivity. High-dose flosulide induced a decrease in glomerular prostanoid production possibly because of COX-1 inhibition. Our results suggest that the therapeutic use of flosulide in proteinuria seems advantageous and deserves further studies because the basal prostaglandin levels remain unchanged in the low-dose–treated group, indicating that the compensatory capacity of prostaglandin production, which is essential for the regulation of renal hemodynamics, is maintained.

Key words: cyclooxygenase, proteinuria, kidney, thromboxane A_2, NSAIDs, renal hemodynamics.

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Nonsteroidal anti-inflammatory drugs (NSAIDs) belong to the most comprehensively investigated drugs. Nevertheless, their use is limited by various side effects. NSAIDs act via inhibition of cyclooxygenase (COX) activity, thereby blocking prostaglandin production. COXs catalyze the conversion of arachidonic acid into prostaglandin H_2 (PGH_2), a precursor for the formation of prostacyclin and thromboxane [1]. Two isoforms exist: a constitutive form (COX-1) that occurs ubiquitously in the body, appearing in the gastrointestinal tract, platelets, and kidney. COX-1 activity fulfills a housekeeping function, that is, it maintains the prostaglandin production necessary for physiological functions [2]. The inducible isoform (COX-2) is expressed in inflammatory conditions and is regulated on the transcriptional level [3, 4]. Most available COX inhibitors act nonselectively [5] in that they inhibit both COX-1 and COX-2 at anti-inflammatory concentrations, especially COX-1 inhibition [6]. Therefore, their therapeutic benefit is limited by gastric and renal toxicity, which in turn appears to be related to the inhibition of prostaglandin production in these tissues [7]. NSAIDs have also been used to treat the nephrotic syndrome [8]. The decline in proteinuria ob-

served in the nephrotic syndrome is assumed to occur at least in part because of a reduction in renal plasma flow, eventually resulting in a reduction in the glomerular filtration rate. The action of NSAIDs on renal plasma flow is at least partially caused by a suppression of COX and COX-dependent metabolites. Therefore, therapeutic application of selective COX-2 inhibitors might result in a more selective inhibition of the inflammatory processes without influencing basal COX-1–dependent prostaglandin synthesis [9].

The aim of this study was to determine the therapeutic efficacy of 6-(2,4 difluorophenoxy-5-methyl-sulfonyl-amino-1-indanone (CGP 28,238), a phenoxyindane-methane-sulfonamide-flosulide, in a model of glomerular nephritis, the passive Heymann nephritis (PHN). A potent anti-inflammatory effect of flosulide is the previously established inhibition of granulocyte function [10]. Two questions were addressed: (*a*) Does flosulide modify glomerulonephritis, that is, the inflammatory process, and (*b*) is this in any way correlated to the inhibition of prostaglandin function?

METHODS

Materials

Flosulide or 6-(2,4 difluorphenoxy-)5-methyl-sulfonyl-amino-1-indanon was provided by Schering (Berlin, Germany), Triton X 100 was purchased from Serva (Heidelberg, Germany) and ethylenediaminetetraacetic acid (EDTA) was purchased from Merck (Darmstadt, Germany). All other chemicals were purchased from Sigma (Deisenhofen, Germany). The anti-Fx1A antibody was a kind gift from Dr. B. Iversen (Bergen, Norway).

Passive Heymann nephritis in rats

All rats were clinically investigated and subjected to standard laboratory tests for four weeks before being sacrificed for kidney removal.

Induction of passive Heymann nephritis in rats. PHN was induced in female Wistar rats (230 to 250 g) by intravenous injection of 1.0 ml of anti-Fx1A antibody prepared and characterized as previously described [11]. The Fx1A antigen represents a tubular antigen of the rat kidney. Anti-Fx1A antibodies were raised in rabbits by intramuscular injection of several probes of Fx1A antigen resolved in "Freud's incomplete-adjuvans" over a period of three months or more. The injection of the anti-Fx1A antiserum then causes an inflammatory process that is localized in the rat glomeruli, the PHN [12].

Experimental procedures. (1) Group A. To assure the effects of PHN on proteinuria, on glomerular prostanoid synthesis, and on COX-1 and COX-2 protein expression, 12 rats were subjected to our study without induction of PHN. These control rats were injected intravenously with equivalent amounts of NaCl solution (0.9%) instead of the Fx1A antibody. Group A animals received no treatment but NaCl solution as placebo.

(2) Group B. PHN was induced in 12 female Wistar rats (230 to 250 g) by intravenous injection of 1.0 ml of anti-Fx1A antibody into the tail vain. Group B animals received no treatment except for a NaCl solution as the placebo.

(3) Groups C and D. Twenty-four PHN rats comprised Groups C and D and were subjected to flosulide treatment. To determine the dose-dependent effect of flosulide on PHN, 12 rats (group C) were treated with 3 mg flosulide/kg body wt/day and 12 rats (group D) received 9 mg flosulide/kg body wt/day. The choice of doses resulted from recommendations of the Schering AG Berlin based on experimental results in animals that were raised by Schering during development of the drug [13].

Electron microscopic diagnosis of PHN. For morphological studies, as early as six hours after the induction of PHN and also at other time points afterwards, several animals were sacrificed and examined to assure there was onset of PHN. Tissue for light microscopy was fixed in 4% formaldehyde, and paraffin sections were stained with periodic acid-Schiff and hematoxylin and eosin stains to evaluate any changes. Electron microscopic changes were detected as electron dense "humps" localized next to the basal membrane in glomeruli.

Schedule of laboratory tests and sample collecting. Therapeutical treatment with flosulide or application of placebo started one week after induction of the PHN and continued for four weeks. In PHN, proteinuria occurred immediately post-induction and reached its maximum level after approximately 14 days.

Once every week during the study, rats were positioned in metabolic cages to collect 24-hour urine samples. Urine creatinine and protein levels were measured using standard laboratory techniques.

Weekly blood samples were taken from the retrobulbar plexus of the animals while they were under ether anesthesia to determine creatinine and protein values in serum for clearance calculation. Blood samples were also collected for determination of thromboxane and prostacyclin in the serum.

On day 28, animals were sacrificed by carbon monoxide poisoning. Kidneys were removed immediately for isolation of glomeruli.

Glomeruli preparation and measurements

Isolation and incubation of glomeruli. Glomeruli were isolated immediately after kidney removal by differential sieving techniques from kidney cortex, and washed three times to remove blood cells. The procedure for glomerular isolation has been described in detail elsewhere [14]. Briefly, glomeruli were centrifuged three times in physiological buffer (5 min, 500 $\times$ *g*). The isolated glomeruli were incubated for one hour in plastic tubes containing

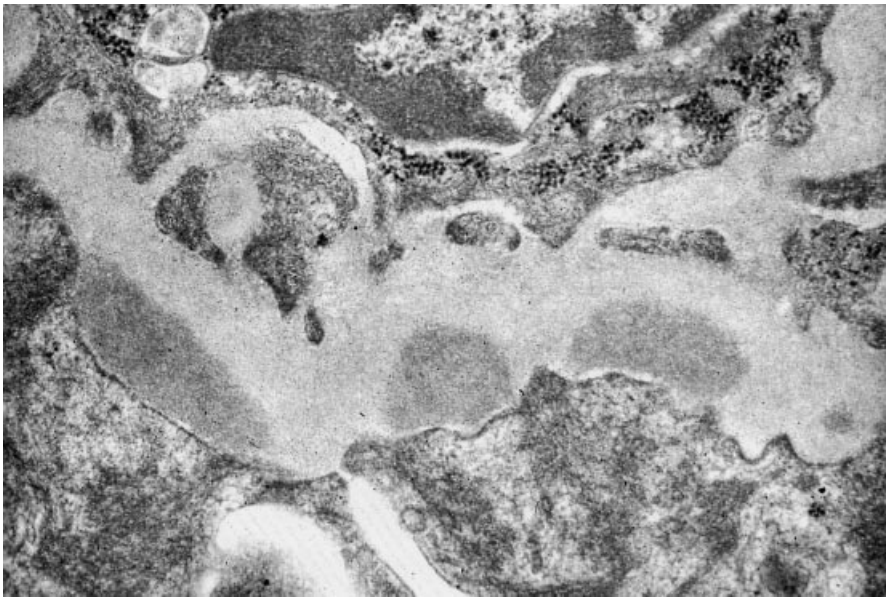

Fig. 1. Six hours after the induction of passive Heymann (PHN) nephritis, 10 animals were sacrificed for morphological examination of their kidneys. By transmission electron microscopy, electron dense "humps" localized next to the basal membrane in glomeruli can be seen as a marker of early onset of PHN.

2 ml of Krebs–Ringer bicarbonate buffer, pH 7.4, at 37°C after the addition of arachidonic acid at a concentration of 50 μmol as substrate for the COXs. To stop the COX reaction, the probes were centrifuged in tubes at 1000 × *g* for 10 minutes at 4°C. The prostanoid levels in the supernatant, which reflected the glomerular prostanoid production, were determined as well as the pellet concentrations of prostanoids for a control. Therefore, the supernatant was collected and frozen until prostanoid determination was performed using radioimmunoassay (RIA) [15]. The glomeruli were solubilized after the addition of lysis buffer [Tris 50 mM, EDTA 10 mM, Triton X 100 1%, phenylmethylsulfonyl fluoride (PMSF) 1 mM, benzamidin 1 mg/ml, pH 7.2] with an ultraturax sonicator. The glomerular protein content was determined according to the method of Bradford [16]. Solubilized glomerular samples were frozen at −20°C for determination of prostanoid content and COX protein expression.

Radioimmunoassay. Prostanoids were determined by direct RIA of the supernatants without prior extraction or chromatographic separation. The supernatant prostanoids thereby reflected the amount of glomerular prostaglandins that were produced by viable glomeruli during one hour of incubation in physiological buffer with sufficient substrate of 50 μmol arachidonic acid. Control measurements after 15 and 30 minutes showed no detectable level of prostanoids. Obviously the glomerular prostanoid production needed a time course of 60 minutes. Prostanoid determinations performed in the solubilized glomerular probes represented the intracellular levels of TxB_2 and 6-keto-$PGF_{1\alpha}$. The assay procedures, sensitivity, and specificity of the antisera used are described elsewhere [15]. TxB_2 was measured as stable metabolite of thromboxane A_2 (TxA_2), and 6-keto-$PGF_{1\alpha}$ was determined as stable degradation product of PGI_2 (prostacyclin).

Western blotting

Cell lysates of isolated rat glomeruli were prepared as described earlier in this article. Equal amounts of cell protein (30 μg) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and dissolved completely by electrophoresis using protein size standards. After protein transfer to nitrocellulose membranes in a semi-dry chamber (4 hr, 30 mA), Western blot analysis was undertaken using commercially available antisera: for COX-1 the monoclonal antibody, PG 19 at 1:500, and COX-2 a polyclonal antibody, PG 26 at 1:1000 (both specific antibodies from Oxford Biochemical, Inc., Oxford, MI, USA). Protein bands were visualized using horseradish peroxidase-linked secondary antibodies and enhanced chemiluminescence. Films were exposed to chemiluminescence approximately 30 minutes before development.

RESULTS

Effect of flosulide on proteinuria in passive Heymann nephritis

Untreated animals with PHN exhibited a significant proteinuria of 71 ± 8 mg/24 hr (group B), whereas the controls (group A) showed a physiological proteinuria of 2.0 ± 0.3 mg/24 hr (Fig. 1). PHN did not reduce total serum protein or serum albumin during the four weeks of the study (data not shown).

Treatment with flosulide significantly reduced the proteinuria in rats with PHN. The animals receiving the lower dose (group C) showed a reduced proteinuria of

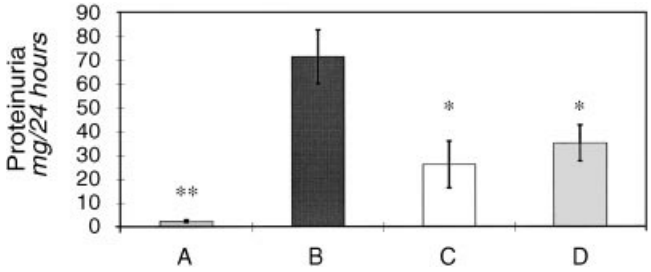

Fig. 2. Proteinuria in the four different study groups. Group A, study controls; group B, rats with PHN; group C, rats with PHN and 3 mg flosulide/kg body wt/day; group D, rats with PHN and 9 mg flosulide/kg body wt/day. *$P < 0.01$ vs. group B. Data are mean ± SD of $N = 12$ animals per group.

26.1 ± 5.8 mg/24 hr as compared with group B, which showed a proteinuria of 71 ± 8 mg/24 hr ($P < 0.05$). Proteinuria in rats with PHN receiving higher-dose flosulide (group D) was also significantly reduced to 35.5 ± 12.9 mg/24 hr as compared with group B animals ($P < 0.05$; Fig. 2). Serum protein was unchanged. Flosulide was able to induce a reduction of proteinuria in rats with PHN at both dosages tested in our study.

Endogenous creatinine clearance in rats with passive Heymann nephritis after treatment with flosulide

The endogenous creatinine clearance (EC_{Cr}) was used as a parameter of renal function. Untreated rats with PHN (group B) showed no significant decrease in EC_{Cr} as compared with controls. During flosulide treatment, there was a decrease of EC_{Cr} from 0.86 ± 0.2 ml/min to 0.6 ± 0.1/min in group C and 0.6 ml ± 0.2/min in group D ($P < 0.01$; Table 1). These results suggest a small but significant nephrotoxic effect of flosulide at both dosages given in the study.

Effect of flosulide on glomerular prostaglandin production in rats with passive Heymann nephritis

Under the addition of arachidonic acid to glomeruli, the glomerular prostanoid production was measured after one hour of incubation in physiological buffer. In comparative determinations of prostaglandins in the rat serum (results not shown here), no change was found in the values associated with PHN or after therapy with the COX-2 inhibitor, flosulide. This indicates that the values determined in our study truly reflect the glomerular *in vivo* production of prostaglandins and that blood contaminations were negligible.

In animals with PHN without treatment (group B), the glomerular TxB_2 levels and the 6-keto-$PGF_{1\alpha}$ levels were significantly increased in the supernatant ($P < 0.05$) as compared with the healthy controls (group A; Fig. 3). Prostanoid concentrations in the glomerular pellet were elevated without reaching significance. In contrast to the elevation of the glomerular prostanoid production, we observed a significant reduction of urine excretion ($P < 0.01$) of the prostaglandin metabolites TxB_2 and 6-keto-$PGF_{1\alpha}$ (Table 2).

Table 1. Endogenous creatinine clearance in rats in this study

Time of determination	Group A	Group B	Group C	Group D
Day 28 *ml/min*	0.86 ± 0.2	0.82 ± 0.3	0.6 ± 0.1[a]	0.6 ± 0.2[a]

Data are ± SD, $N = 12$.
[a] $P < 0.01$ vs. group A

During treatment with flosulide (3 mg/kg body wt/day, group C), the glomerular prostaglandins in the supernatant were also significantly elevated ($P < 0.01$). Pellet prostaglandin concentrations were also elevated, but this alteration did not reach significance. On 3 mg flosulide, the urine excretion of prostaglandins was significantly decreased ($P < 0.01$).

During treatment with high-dose flosulide (group D), there was no elevation in glomerular prostanoid production, and pellet concentrations of prostanoids were reduced significantly. TxB_2 was as low as 1300 ± 290 pg/ml, and 6 keto-$PGF_{1\alpha}$ was reduced to 1040 ± 180 pg/ml ($P < 0.05$). The reduction of urine excretion was also observed after administration of 9 mg flosulide ($P < 0.01$).

The results showed that glomerular prostaglandins in the supernatant were significantly elevated in PHN and remained high after treatment with low-dose flosulide. High-dose flosulide therapy induced a reduction in glomerular prostanoid production and a significantly reduced prostanoid content in the pellet. Summarizing the data, it is suggested that only high-dose COX-2 inhibition with flosulide alters glomerular prostanoid production, whereas low-dose flosulide does not change glomerular prostanoid production.

Expression of COX-1 protein

The protein band of the constitutive COX, COX-1, was detected in the glomerular protein samples with a specific monoclonal antibody as a 70 kDa band (Figs. 4 and 5). COX-1 expression did not quantitatively differ between the controls and untreated rats. The COX-1 protein expression did not change after low-dose therapy with flosulide (3 mg flosulide/kg body wt/day), while the high-dose therapy (9 mg flosulide/kg body wt/day) suppressed COX-1 protein expression completely.

The protein bands were directly compared with each other. Because the amount of protein was according to an "all or nothing" rule, which meant that either 100% or almost no protein was detectable, the quantitation by a luminescence integral determining method was not necessary.

Expression of COX-2 protein

The specific protein of the inducible COX, COX-2, was detected in the glomerular protein lysates as a 71

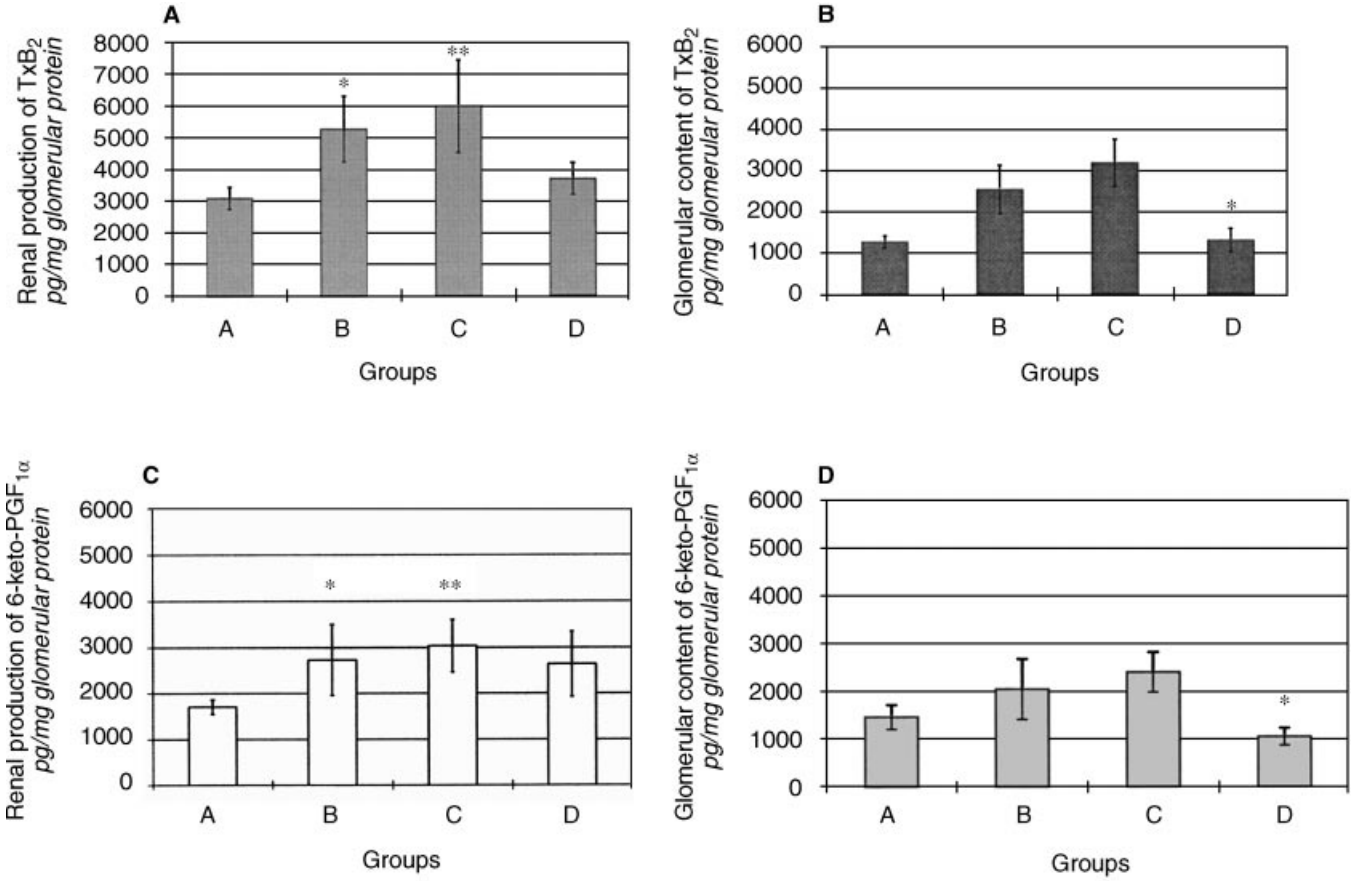

Fig. 3. Determination of the stable metabolites of the COX-dependent prostaglandins thromboxane A (TxB_2) and prostacyclin (6-keto-$PGF_{1\alpha}$) by means of RIAs. Isolated glomeruli were incubated in Krebs-Henselein buffer (37°C, pH 7.2) for 60 minutes after the addition of sufficient substrate (50 μmol arachidonic acid) to measure renal prostanoid production. After termination of the cyclooxygenase reaction by centrifugation (1,000 * *g*, 10 min), samples of supernatant were collected, and glomeruli were solubilized. In both fractions, TxB_2 and 6-keto-$PGF_{1\alpha}$ were determined (X ± SEM; $N = 12$). Significance was calculated according to the Mann-Whitney U-test in comparison with group B: $^*P < 0.05$, $^{**}P < 0.01$.

Table 2. Effects of flosulide on renal prostanoid production and urine excretion of prostanoids

	Renal prostanoid production *pg/mg glomerular protein*		Urine excretion of prostanoids *ng/g creatinine excretion*	
	TxB_2	6-keto-$PGF_{1\alpha}$	TxB_2	6-keto-$PGF_{1\alpha}$
Group A	3073 ± 355	1702 ± 161	308 ± 36	246 ± 22
Group B	5255 ± 1041	2724 ± 770	177 ± 18	137.5 ± 11
P	< 0.05	< 0.05	< 0.01	< 0.01
Group C	5975 ± 1465	3028 ± 573	82 ± 8.2	86 ± 31
P	< 0.01	< 0.01	< 0.01	< 0.01
Group D	3686 ± 499	2633 ± 714	83 ± 11	79 ± 4.7
P	NS	NS	< 0.01	< 0.01

Concentrations were determined by radioimmunoassay. Data are shown as ± SD, $N = 12$. *P* values are compared with group A. NS is not significant.

kDa band using a specific antiserum (Fig. 3). Comparable results for vascular smooth muscle cells of the rat are in literature [17]. Furthermore, the amount of detected protein could be compared with the constitutively expressed COX-1 isoform.

In the glomeruli of group A (12 control animals), only a narrow band was demonstrated after a long exposure of the films to chemiluminescence (data not shown) that reflected the basal expression of COX-2. In the glomeruli of group B (12 rats with PHN; Fig. 3), a strong induction of COX-2 during PHN was found. The markedly elevated protein expression compared with the controls was clearly evident on day 14 of the study.

Cyclooxygenase 2 protein expression in group C rats

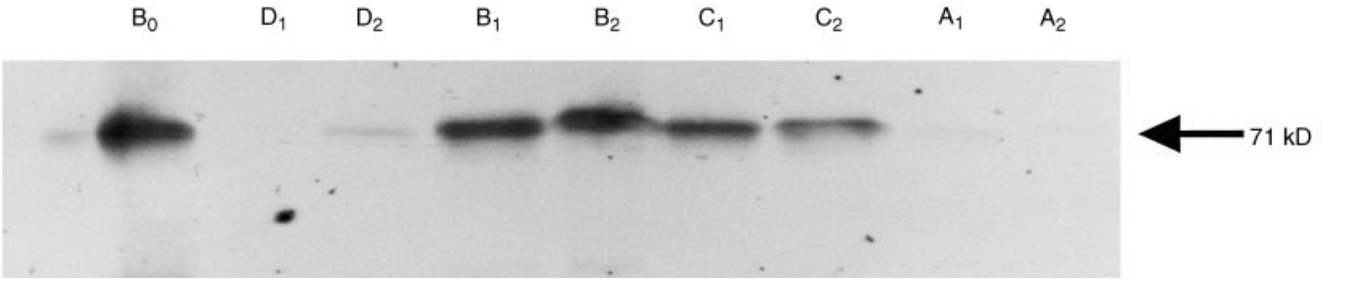

Fig. 4. Western blots showing the specific detection of COX-2 as a 71 kD protein band in solubilized rat glomeruli. All lanes contain a total of 30 μg protein of the glomerular probes that were solubilized according to the experimental protocol described in the **Methods** section. Probes of the corresponding group ($N = 12$) showed comparable results. B_0 is a protein sample of group B (rats with PHN) on day 14; D_1 and D_2, protein samples of group D (rats with PHN, 9 mg flosulide/kg body wt/day) on day 28; B_1 and B_2, protein samples of group B (rats with PHN, no treatment) on day 28; C_1 and C_2, protein samples of group C (rats with PHN, 3 mg flosulide/kg body wt/day) on day 28; A_1 and A_2 are protein samples of group A (controls) on day 28.

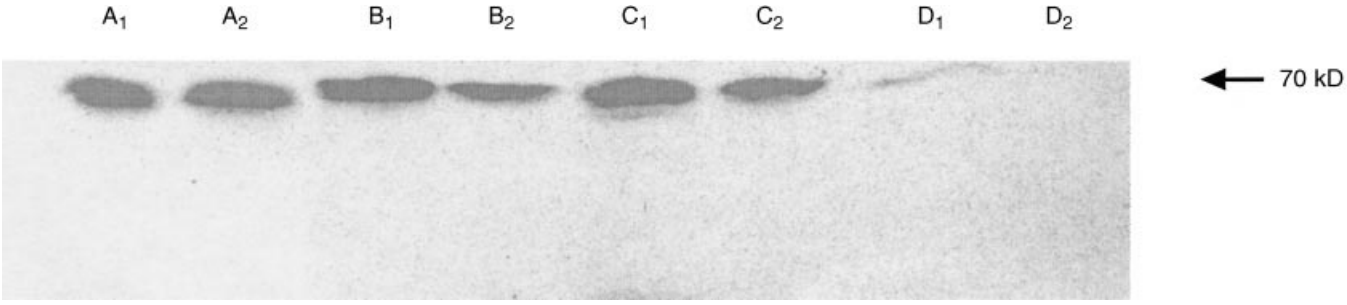

Fig. 5. Western blots showing the specific detection of COX-1 as a 70 kD protein band in solubilized rat glomeruli. All lanes contain a total of 30 μg protein of the glomerular probes that were solubilized according to the experimental protocol described in the **Methods** section. Probes of the corresponding group ($N = 12$) showed comparable results. A_1 and A_2 are protein samples of group A (controls) on day 28. B_1 and B_2 are protein samples of group B (rats with PHN, no treatment) on day 28. C_1 and C_2 are protein samples of group C (rats with PHN, 3 mg flosulide/kg body wt/day) on day 28. D_1 and D_2, protein samples of group D (rats with PHN, 9 mg flosulide/kg body wt/day) on day 28.

(low-dose flosulide therapy for PHN) was comparable to the protein expression in untreated animals (group B). In contrast, COX-2 protein expression was markedly suppressed in rats treated with high-dose flosulide (group D).

Apparently, an up-regulation of COX-2, the inducible isoform of COXs, occurred in conjunction with PHN, compared with an only slightly pronounced basal expression. At the higher dose (9 mg/kg body wt/day), the flosulide-induced inhibition of the enzyme caused a reduction in the protein expression of COX-2, whereas protein expression on 3 mg flosulide/kg body wt/day remained approximately the same.

DISCUSSION

Little information exists on the pharmacological action of COX-2–selective inhibitors in the kidney [18]. The availability of a COX-2 selective inhibitor encouraged us to examine its potential therapeutic effect on inflammatory processes in the kidney. The aim of this study was to investigate the therapeutic action of 6-(2, 4) difluorophenoxy-5-methyl-sulfonylamino-1-indanone (CGP 28,238), a phenoxyindane-methane-esulfonamide named flosulide, in PHN. This investigation was conducted *in vivo* to test if the selective COX-2 inhibitor (flosulide) affected renal function, COXs, or renal prostaglandin production. PHN in the rat was used as an animal model. The renal histology was investigated once by the use of electron microscopy to prove the early onset of PHN after injection of the anti-FxA1 antibody to the animals. Structural changes in rat kidney after therapy with a highly selective COX-2 inhibitor might correlate with biochemical and physiological findings, but this was not investigated further.

In the kidney, prostanoids are produced mainly in the medulla, in the ascending loop, and in the cortex. Renal COXs participate in the regulation of renal blood flow, sodium and water reabsorption, and the release of renin. All of these functions are mainly regulated by COX-1, but the release of renin may also be regulated by COX-2 [19]. COX-2 has been detected in the kidney as well [20], and its activation probably represents an important compensatory mechanism for maintaining sufficient blood flow in the kidney during pathogenic processes [7]. PHN represents this type of critical inflammatory

situation in the kidney, and is characterized by the rapid production of local prostaglandins as well as other features. This takes place directly following injection of the irritating antibody directed against the tubular antigen Fx1A [11, 12].

Previous studies showed that all mice with a knockout of all COX-2 isoenzymes develop renal dysplasia and half of them develop cardiac fibrosis [21]. The lack of COX-2 did not in any way affect the inflammatory capacity of their immune systems. Although not all reasons for those circumstances are yet fully understood, it has become clear that COX-2 is essential for physiological functions and that a total inhibition of COX-2 action might not be favorable toward continued health.

Investigations on the detailed function of COX-1 and COX-2 in the kidney are not yet complete. COX-2 has been found to be up-regulated in inflammatory conditions. Therefore, the therapeutic use of COX-2 inhibitors as specific anti-inflammatory agents was considered [22]. Investigations in the stomach have shown that COX-2 inhibitors exhibit minor toxicity while maintaining their anti-inflammatory action [23]. Comparable results have not been previously found in the kidney [6]. Therefore, it seemed plausible to inhibit COX-2 as a modulator of inflammation, while the supply of prostanoid precursors by COX-1 could remain undisturbed.

In our study, rats with PHN developed proteinuria and elevated glomerular prostaglandin synthesis, which is concordant with previous studies [24]. Despite the increasing evidence for enhanced intrarenal production of eicosanoids in a number of experimental as well as clinical forms of glomerulonephritis, the cause and cellular source of these products remain controversial. These cells were either infiltrating blood elements such as leukocytes, monocytes, and platelets, recruited and activated to synthesize eicosanoids at the site of glomerular injury, or were glomerular epithelial and mesangial cells. The latter intrinsic glomerular cells could possibly be activated by a number of inflammatory factors. Alterations in anionic structures of the glomerulus as well as increased renal eicosanoid synthesis have been described in glomerular disease. Unlike other mediators, eicosanoids formed within the kidney are not stored following synthesis, but are either locally metabolized or removed from the kidney in the lymphatic and venous drainage or excreted into the urine [8].

In parallel experiments we measured the glomerular production of the stable prostaglandin metabolites TxB_2 and PGE_2 (reflecting TxA_2 and prostacyclin as active prostaglandins) in two compartments of the kidney, in the protein lysates of the glomeruli, as well as in the supernatant of the incubated glomeruli after the removal of blood cells by careful washing of the glomeruli. Furthermore, we measured the urinary excretion of the prostaglandins in all four therapeutic rat groups.

In our study, all urinary prostaglandin levels were very low, although the measured glomerular prostaglandins reached quite high levels. This was already the case in the study of Heise et al, which concerned flosulide treatment of rats with PHN until day 14 [25]. As Ciabattoni and Pugliese reported [8], the urinary excretion of prostaglandins reflects the situation of the whole kidney production, including tubular cells, mesangial cells, and glomerular cells. Our data suggest that the urinary excretion of prostaglandins does not completely reflect the overall prostaglandin production in PHN, which is obviously focused on the glomeruli. This coincides with the fact that the PHN represents an inflammatory disease of the glomeruli in first line with a severe glomerular injury. Urinary prostaglandins were shown to be unsuitable to study the effect of a COX-2 inhibitor on the course of prostaglandin production in PHN.

In contrast, the glomerular prostaglandins, which are highly elevated in PHN [24], remained high during therapy with low-dose flosulide. Enhanced prostaglandin synthesis of the glomeruli was associated with elevated COX-2 protein expression, but not with an elevation of COX-1 protein expression. COX-1 protein expression remained unchanged and was easily detectable in both the controls and the animals suffering from PHN and not receiving therapy. Apparently, an inflammatory stimulus did not change the expression of the constitutive isoenzyme. The increase in glomerular prostaglandins in conjunction with PHN thus appeared to be COX-2 dependent.

Therapy with flosulide induced a significant reduction in proteinuria. This effect occurred in a dose-independent manner in the two dosage groups. These results suggested that selective COX-2 inhibitors possess an anti-inflammatory, immunomodulatory effect in inflammatory processes of the kidney. The remaining prostanoid production is generally believed to be essential for the maintenance of glomerular filtration rate in critical inflammatory conditions of the kidney [7]. In this study, a nonspecific, nephrotoxic effect of flosulide had to be excluded as a possible explanation for the antiproteinuric action of the drug. We evaluated renal function in all therapy groups in terms of EC_{Cr} and compared them with each other. A small though significant reduction in EC_{Cr} to 0.6 ml/min compared with 0.86 ml/min in controls was shown on flosulide. Thus, it can be assumed that a certain nephrotoxicity existed in both groups. After all, the reduced clearance performance of the kidney alone is not enough to explain the differences in protein expression and prostaglandin production found between the two dosage groups, but may support the antiproteinuric action.

In contrast to its antiproteinuric action, the effect of flosulide on glomerular prostaglandin production and on the protein expression of COX-2 was dependent on the dose administered. Low-dose flosulide therapy did not lead to any major changes in renal prostaglandin production

or in COX-2 protein expression, whereas COX-1 protein expression remained unchanged.

A marked effect was shown with 9 mg flosulide/kg body wt/day, as there was a significant reduction in prostaglandin production, although the TxA_2 levels were more affected than the prostacyclin content. In addition to this, suppression of COX-2 protein expression was also observed on high-dose flosulide therapy. The suppression of COX-1 protein expression after high-dose flosulide administration indicates that the drug's action is nonselective within this dose range. This observation was also made by other authors [9, 20, 26]. The drop in prostaglandins on high-dose flosulide therapy might therefore be induced by an additional impairment of COX-1.

When studied by Western blotting, both COX-1 and COX-2 protein were not detectable when animals were treated with high-dose flosulide. Possible explanations of this finding remain speculative, because detailed examinations of possible transcriptional or translational mechanisms are missing. There are only few studies on the molecular signal pathway of the COX-2 expression. One recent publication reports that TLCK, a serine protease inhibitor (Nalpha-P-tosyl-L-lysine chloromethyl ketone, 100 μmol/liter) and MG 132, a proteasome complex (26S) inhibitor (10 μmol/liter), inhibited interleukin-1β–induced COX-2 enzyme activity (PGE_2 formation) and COX-2 gene expression at the level of mRNA and protein in rat mesangial cells [27].

Because the expression of both COX enzymes 1 and 2 are blocked under high-dose flosulide, it appears to be likely that high-dose flosulide leads to an inhibition of protein synthesis of COX-1 and COX-2. By which mechanisms this occurs remains unclear. It is possible that negative feedback mechanisms play a role, but it is also likely that high-dose flosulide might be toxic for renal cells and might affect renal cell viability in general. This hypothesis agrees with the fact that flosulide has been removed from the market because of its nephrotoxicity [9].

The fact that the prostaglandins remained high on low-dose flosulide could suggest that COX-1 fulfills the function of maintaining the synthesis of prostaglandin precursors while COX-2 is inhibited. On the other hand, it has to be considered whether flosulide inhibition of COX-2 is complete. The selectivity of flosulide has been shown by Klein et al [20], who studied COX-2 induction in rat mesangial cells by immunoblot analysis. In parallel experiments, human platelets, known to host only COX-1, were taken to study inhibitory effects on the constitutive enzyme; these experiments showed that flosulide inhibits COX-1 in much higher concentrations than other NSAIDs such as indomethacin or diclofenac. The IC50 of flosulide in that study was about 50,000-fold higher than the IC50 value of indomethacin. In contrast to these results, the inhibitory effect of flosulide compared with indomethacin and diclofenac was approximately equal with regards to COX-2 in rat mesangial cells. Summarizing, Klein et al showed that for flosulide, the ratio of $IC50_{COX\text{-}2}/IC50_{COX\text{-}1}$ amounts to 5000, demonstrating a high selectivity of this drug for COX-2.

In conclusion, we observed that when low-dose flosulide was used, the proteinuria in PHN was reduced, but the elevated prostaglandin levels remained unaffected. COX-1 and COX-2 protein expression remained unchanged. With high-dose flosulide, proteinuria was still reduced, but renal prostaglandin levels were also reduced. COX-1 and COX-2 protein expression was highly suppressed.

There is actually only one comparable study on COX-2 inhibitors in the kidney [28]. Hirose et al showed that in the anti–Thy-1 glomerulonephritis, the COX-2 protein was induced on days 4 and 10 during but not in the glomeruli of control rats. COX-2 was localized exclusively in glomerular epithelial cells, as shown in our study in PHN. In contrast to our results, the COX-1 protein in Hirose's study was not or only faintly detected in rat control glomeruli, which could be due to the localization (shown by immunofluorescence) along glomerular capillary walls but not directly in the glomeruli. That study actually shows no comparable investigations on the effect of COX-2 inhibitors on glomerular prostaglandin production.

Although the investigations concerning the detailed function of COX-1 and COX-2 in kidney are not yet finished, our results from experiments in rats clearly indicate that the use of a COX-2–selective inhibitor such as flosulide in a low-dosage range represents an interesting immunomodulatory therapeutic concept. Its benefit is probably based on the fact that the low-dose application of flosulide leaves the supply of prostanoid precursors in the glomeruli untouched. This important compensatory mechanism of the kidney seems to be essential for the maintenance of renal perfusion in critical inflammatory situations of the kidney. COX-2 inhibitors are also discussed as anticancer agents as well as in preventing premature labor, and perhaps even as a factor retarding the progression of Alzheimer's disease [18]. In any case, whether or not the therapeutic application of COX-2 inhibitors in patients with glomerulonephritis over a longer period of time might be beneficial remains an interesting subject for further investigation [13].

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Reprint requests to Cornelia Blume, M.D., Klinik für Nephrologie und Rheumatologie, Medizinische Einrichtungen der, Heinrich-Heine Universität Düsseldorf, Postfach 101007, 40001 Düsseldorf, Germany.

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