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Role of enhanced glomerular synthesis of thromboxane A_2 in progressive kidney disease

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Role of enhanced glomerular synthesis of thromboxane A_2 in progressive kidney disease. Normotensive rats of the Milan strain (MNS) spontaneously develop focal glomerulosclerosis. In order to explore the contribution of glomerular thromboxane (TX) A_2 synthesis to the development of the disease, we have characterized the time course of renal functional and biochemical changes, and their modification by long-term treatment with a TX-synthase inhibitor. Oral administration (150 mg · kg^{-1} from 1 to 14 months of age) of FCE 22178 suppressed enhanced glomerular TXB_2 production at all experimental times (mean inhibition 80%) and proteinuria (varying between 27.1 and 73.0%) while preserving renal blood flow and glomerular filtration rate. These effects of TX-synthase inhibition were seen in the absence of any statistically significant changes in systemic blood pressure. Moreover, FCE 22178 had no antihypertensive effects in hypertensive rats of the Milan strain (MHS) nor in spontaneously hypertensive rats (SHR). Treatment also prevented the age-related hypoalbuminemia and hyperlipidemia observed in control MNS and significantly ($P < 0.01$) reduced glomerular histologic damage, as demonstrated by light microscopy studies and measurement of sclerotic area. We conclude that: 1) MNS rats provide an animal model of long-lasting proteinuria characterized by an age-related increase in glomerular TXB_2 production paralleled by progressive loss of renal structural integrity and function and by a secondary dyslipidemia; 2) pharmacological inhibition of glomerular TX-synthase attenuates the structural as well as the functional expression of kidney disease, without a primary effect on systemic blood pressure. These data are suggestive of an important modulating role of TXA_2 in the progression of MNS renal disease.

The Milan hypertensive strain (MHS) and the Milan normotensive strain (MNS) are genetically related as they were both developed from common ancestor Wistar rats by two-way selection and inbreeding initiated in 1965 [1].

Several lines of evidence obtained both in vitro and in vivo suggest that the kidney plays a pivotal role in the development of hypertension in MHS [2, 3]. However, unlike the spontaneously hypertensive rats of the Okamoto strain (SHR), MHS neither display renal lesions and proteinuria when hypertension is fully established, nor develop glomerulosclerosis with age. In contrast, it was recently found [4] that the corresponding normotensive controls, MNS, do develop focal glomerulosclerosis. Thickening of the glomerular basement membrane, capillary lumen obliteration and pathological changes of podocytes with extensive fusion of the foot processes are the prominent features of the disease at 16 months; at this age marked proteinuria occurs [4].

Similar age-dependent glomerulosclerosis has been described previously in other normotensive inbred rat strains, although usually at an older age [5]. Various factors have been proposed to influence the occurrence of this disease such as sex, diet and immunologic injury.

In the MNS, however, there is no evidence of immunologic damage, as shown by immunofluorescence microscopy [4]. On the other hand, preliminary studies have shown that glomeruli isolated from adult MNS, produce significantly more thromboxane (TX) A_2 (measured as TXB_2) in vitro than glomeruli from age-matched MHS [6].

Several experimental [7–10] as well as clinical [11] forms of glomerular disease share alterations in intrarenal arachidonate metabolism, the most prominent feature of which is represented by enhanced intrarenal production of TXA_2.

This biochemical abnormality is often associated with proteinuria, deteriorating renal function and progressive glomerulosclerosis, and suggests important effects of locally generated TXA_2 on glomerular arterioles and mesangial cells [12]. A causal relationship between enhanced intrarenal TXA_2 biosynthesis and functional and structural changes has been established in several experimental models through the use of selective TX-synthase inhibitors [7–10, 13, 14]. Thus, the aims of the present investigation were: 1) to further characterize the development of renal disease in MNS, as a potential model of heavy and persistent proteinuria and spontaneous age-related glomerulosclerosis; 2) to characterize the spectrum of lipid abnormalities accompanying progressive renal damage; 3) to explore the role of glomerular TXA_2 biosynthesis in this model through serial biochemical measurements and long-term treatment with a TX-synthase inhibitor.

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Our findings demonstrate a role for enhanced glomerular TXA_2 in contributing to renal functional and structural abnormalities in this model by showing that these can be substantially prevented by pharmacologic blockade of TXA_2 biosynthesis.

Methods

Experimental groups

Long-term studies. Male MHS and MNS rats of the F54 generation (42 animals from each strain), aged four weeks and with body weight ranging between 70 and 80 g at the start of the experiment, were used for this study. All rats received a standard chow diet (Altromin MT, A. Rieper Vandois, Italy) containing 0.3% Na^+ and 23% protein, and had free access to food and water throughout the experiment. The animals of each strain were divided in two groups ($N = 21$).

Group 1 (MNS rats) and group 2 (MHS rats) were treated with the selective inhibitor of TX-synthase, FCE 22178 [5,6-dihydro-7-(1H-imidazol-1-yl)-2-naphtalene-carboxylic acid]. FCE 22178 Na-salt (Batch A05001) was synthesized in Farmitalia Carlo Erba Research Laboratories (Milan, Italy) [15]. The daily dose of the drug was 50 mg · kg^{-1} given orally by gastric tube in the morning plus 100 mg · kg^{-1} dissolved in the drinking tap water. The concentration of the drug solution was adjusted every second day according to water intake, in order to maintain the dosage constant throughout the experiment. This treatment schedule was found adequate, in preliminary experiments, to maintain over 24 hours greater than 80% suppression of circulating platelet TX-synthase activity (as reflected by TXB_2 levels generated in whole blood during clotting [16]).

Group 3 (control MNS) and group 4 (control MHS) received saline, (0.2 ml · 100 g^{-1} body wt). All animals were treated as above from 1 to 14 months of age. Water consumption, food intake and body weight were measured daily between 8 and 10 a.m., just before drug administration by gastric tube.

Systolic blood pressure (SBP) measurements and urine collections were performed bimonthly in all MNS and MHS rats.

At 4 weeks, and then at 2, 8 and 14 months of age six MNS and MHS rats randomly chosen out of the initial number were used for renal clearance studies, blood lipid and albumin measurements. At the end of clearance studies, kidney specimens were processed for light and electron microscopy and glomeruli isolated for measurement of TXB_2 production.

In order to investigate whether the effect of TX-synthase inhibition on renal hemodynamics might be secondary to an effect of the inhibitor on systemic arterial blood pressure, the effect of FCE 22178 on this parameter was also studied in four additional groups of rats of the Okamoto strain. Group 5 (spontaneously hypertensive rats, SHR, $N = 21$) and group 6 (Wistar Kyoto normotensive rats, WKY, $N = 21$) were given FCE 22178 following the treatment schedule reported above; group 7 (control SHR, $N = 21$) and group 8 (control WKY, $N = 21$) were given saline. All animals were four weeks old at the beginning of the experiment.

Acute studies. The role of TXA_2 in modulating renal hemodynamics was further explored in separate groups of 12-month-old MNS rats, that is, at a stage when their renal disease is fully developed [4].

We compared the effects of an intravenous infusion of FCE 22178 (10 mg · kg^{-1} · hr^{-1}; $N = 9$) to that of the TXA_2/PGH_2 receptor antagonist BM 13,177 (3 mg · kg^{-1} · hr^{-1}; $N = 6$) on the basis of the assumption that in presence of TXA_2-synthase inhibition, the accumulating PGH_2 might act as TXA_2 agonist. The possible modulating role of enhanced vasodilator prostaglandin synthesis, caused by FCE 22178 through re-direction of PGH_2 metabolism, was studied in a further group of MNS ($N = 6$) receiving both compounds.

The selected infusion rate of FCE 22178 was able to completely (> 90%) inhibit TXB_2 formation in whole blood during clotting, as determined in preliminary experiments. Similarly, the chosen infusion rate of BM 13,177 completely suppressed the response to bolus injections of the TXA_2 mimetic U46619 (2 $\mu g \cdot kg^{-1}$). Control, saline infused, 12-month-old MNS ($N = 5$) and normal Sprague-Dawley rats (3 months of age; $N = 8$) were included in the study for comparison.

Experimental procedures

Systolic blood pressure measurement and urine collection. SBP was measured in conscious rats of the Milan and of the Okamoto strains bimonthly (first measurement at 4 weeks of age, before starting the treatment) by the indirect tail-cuff method using a W+W BP recorder (Basile, Varese, Italy) after prewarming the animals at 29°C for 10 minutes. Each value represents the mean of at least five consecutive readings.

Urine collections were made one week after SBP measurement. The animals were placed in metabolic cages with free access to food and water. After an adaptation time of three days, two consecutive 24-hour urine samples were collected into ice cooled containers.

Total urinary protein concentration was measured by the Biuret method (Total protein, Boehringer M., Mannheim, FRG) and the excretion rate expressed as mg · 24 hr^{-1}.

For each parameter, the mean of the two 24-hour collection periods was calculated and used for further calculations and statistics.

Renal clearance in chronic studies. Under light halothane anesthesia the right carotid artery and the left jugular vein were cannulated with a polyethylene tube (PE 50) for blood sampling and infusion, respectively. Glomerular filtration rate (GFR) and renal plasma flow (RPF) were measured four hours after surgery in conscious rats according to the constant infusion method [17]. Briefly, after a priming dose (0.4 ml · 100 g^{-1} body wt) of 12 $\mu Ci \cdot kg^{-1}$ ^{125}I-hippuran, (Amersham Int. plc., Buckinghamshire, UK) and 24 $\mu Ci \cdot kg^{-1}$ ^{51}Cr-EDTA (Amersham), a maintenance infusion of 18 $\mu Ci \cdot kg^{-1} \cdot hr^{-1}$ of ^{125}I-hippuran and 36 $\mu Ci \cdot kg^{-1} \cdot hr^{-1}$ of ^{51}Cr-EDTA were delivered into the venous catheter by a syringe (Hamilton Bonaduz AG, Bonaduz, Switzerland) fitted into an infusion pump (model 341, Sage Instruments, division of Orion, Cambridge, Massachusetts, USA) for three hours. Three duplicate samples of plasma (20 μl) were collected at 20 minute intervals starting after 90 minutes of equilibration and counted in a Beckman gamma 8000 Counting system (Beckman Instruments Inc, Irvine, California, USA) for 20 minutes. GFR and RPF were calculated by standard formulae. At the end of the clearance studies, the animals were anesthetized by intraperitoneal injection of Na pentobarbital (45 mg · kg^{-1}) and their kidneys were removed; specimens were obtained for histologic examination. The remaining tissue was immediately frozen and stored at −80°C, until processed for the isolation of glomeruli in order to assess their ability to synthe-

size TXA_2 ex vivo. Preliminary experiments showed that freezing does not alter glomerular TXB_2 production (data not shown).

Renal clearance in acute studies. Renal function in conscious 12-month-old MNS rats was measured by the clearance of inulin (C_{In}) (E. Merck, Darmstadt, FRG) and p-aminohippuric acid (C_{PAH}) (Sigma Chemical Co., St. Louis, Missouri, USA). Rats were chronically implanted with indwelling arterial and venous catheters 48 hours in advance under halothane anesthesia for blood collection and drug infusions; the day of the experiments a large polyethylene tube (PE90) was inserted into the bladder through a small suprapubic incision for urine collection.

For comparison, control, three-month-old Sprague-Dawley rats were similarly prepared.

Two hours after surgery, a priming i.v. dose of 5% inulin and 0.2% PAH was given, followed by a slow infusion of saline (2 $ml \cdot hr^{-1}$) containing sufficient inulin and PAH to maintain, both in normal and nephrotic rats, their plasma levels constant (0.8 to 1.3 mg/ml for inulin and 0.005 to 0.008 mg/ml for PAH, respectively).

A 60-minute equilibration period was allowed, and a baseline clearance period was obtained (60 min), then animals received the different i.v. infusions (0.5 $ml \cdot hr^{-1}$). Four 60-minute clearance periods were then performed. Plasma and urine inulin and PAH were determined using colorimetric standard techniques [18, 19]; C_{In} and C_{PAH} were corrected per gram of wet kidney weight.

Measurement of serum lipids and serum albumin. Blood samples were obtained in fed conditions from the venous catheter of MNS and MHS rats undergoing clearance studies at 1, 2, 8, 14 months of age. Serum total cholesterol, triglycerides and phospholipids were determined by enzymatic colorimetric methods (Clinicals, Carlo Erba, Milan, Italy) using a Kem-O-Mat 2 autoanalyzer (Coulter Kontron, Milan, Italy). Serum albumin was measured by rocket immunoelectrophoresis [20] in control and treated 14-month-old MNS. Rat albumin (Cohn fraction V) purchased from Sigma Chemical Co. was used as a standard for albumin determinations.

Glomerular isolation and incubation. Glomeruli were isolated as previously described [9] with minor modifications. Briefly, the renal capsules were removed, the cortex was separated from the medulla and finely minced to a paste-like consistency. All subsequent preparative steps were performed at 4°C.

The homogenate was gently passed through a 106 μm stainless steel sieve which excluded the tubules and then washed with Krebs-Ringer phosphate buffer (KRB, 120.3 mM NaCl, 4.8 mM KCl, 1.2 mM $MgSO_4$, 15.6 mM, pH 7.4) over a 75 μm sieve which retained the glomeruli. The glomeruli were suspended in ice cold KRB and allowed to settle spontaneously. The supernatant was discarded and the pellet resuspended in the same buffer solution. This washing step was repeated two more times. The purity of isolated glomeruli was determined microscopically by counting the number of glomerular and non-glomerular particles suspended in a given volume. The final pellet consisted of decapsulated glomeruli with less than 5% tubular contamination. For direct radioimmunoassay (RIA) measurements of TXB_2 isolated glomeruli were centrifuged at 150 × g for five minutes, resuspended in 1 ml KRB supplemented with 2.57 mM $CaCl_2$, and incubated for 60 minutes in a Dubnoff shaking water bath at 37°C. Incubation was stopped by centrifugation at 3,000 × g for five minutes and the supernatant of each tube was collected and frozen until assayed for TXB_2. Glomerular protein concentration was determined according to the method of Lowry et al [21].

Glomerular TXB_2 radioimmunoassay. TXB_2, the stable breakdown product of TXA_2, was assayed in glomerular supernatants at a final dilution of 1:150. RIA was performed as previously described [13]. Briefly, 5,000 dpm of ^3H-TXB_2 (113 $Ci \cdot mmol^{-1}$) and an aliquot of specific rabbit antiserum (final dilution 1:250,000) sufficient to bind 40 to 50% of the tritiated compound were incubated for 16 to 24 hours at 4°C in a final volume of 1.5 ml for each assay tube. Separation of antibody-bound from free-labeled antigen was achieved by rapid addition of 0.1 ml of human prostaglandin-free plasma and 0.1 ml of a charcoal suspension (50 $mg \cdot ml^{-1}$) and subsequent centrifugation at 4°C. The supernatant solution containing antibody-bound TXB_2 was decanted directly into 8 ml of Instagel (Packard Instrument Co. Inc., Downers Grove, Illinois, USA). Radioactivity of samples was counted in a liquid scintillation counter (LS 1800 model, Beckman Instruments, Irvine, California, USA). Results were expressed as $ng \cdot mg^{-1}$ protein. The smallest concentration of TXB_2 that could be measured with 95% confidence was 2 $pg \cdot ml^{-1}$. The cross reactivity at 50% displacement of other arachidonate metabolites was as follows: 2,3-dinor-TXB_2, 10.5%; 6-keto-$PGF_{1\alpha}$, <0.006%; $PGF_{2\alpha}$, 0.023%; PGD_2, 0.02%; PGE_2, 0.007%; arachidonate, <0.004%.

Interassay and intraassay variability, evaluated by assays of several glomerular preparations, averaged 4% and 3%, respectively, over a range of concentrations from 1 to 100 $pg \cdot ml^{-1}$. Validation of RIA measurements was obtained by several independent criteria: dilution and recovery studies, comparison among multiple antisera, characterization of the chromatographic pattern of distribution of the extracted thromboxane-like immunoreactivity on thin layer chromatography and comparison with gas chromatographic-mass spectrometric determinations. These techniques are described in detail elsewhere [11].

Morphology. The kidneys from 82 out of 84 animals (42 MHS and 40 MNS, both treated and untreated) were processed according to following schedule: after sacrifice, 2-mm thick slices from each kidney were postfixed in neutral 10% formalin, dehydrated and embedded in metacrylic resin (Historesin, LKB, Bromm, Sweden); 0.5 μm sections were stained with hematoxylin/eosin (H/E), periodic acid Schiff (PAS) and periodic acid Schiff + methenamine (PASM), for light microscopy.

Light microscopic studies included histopathological observations with emphasis on the degree of glomerular involvement by sclerosis.

Sclerosis was defined as an increase in mesangial matrix, or segmental substitution of the capillary tuft by fibrosis, or adhesion between visceral and parietal layers of epithelial cells, with various degrees of capillary lumen obliteration. A PAS stained, 5-μm thick coronal section from each animal was blindly estimated by two independent observers and the degree of glomerulosclerosis was semiquantitatively graded from 0 (absent) to 4 (global hyalinosis). Grade 1 to 4 was given when the glomerular area involved was 25%, 50%, 75% and 100%, respectively. The extent of glomerulosclerosis was estimated as the percent of sclerotic area over the total glomerular area; for

example, if 25 of 110 glomeruli had a lesion of 1+ and 10 of 110 had a lesion of 3+ the final percent of affected area in this sample would be: $[(25 \times 1/4) + (10 \times 3/4)] \times 100/110 = 12.5\%$. The adopted procedure is a modification of that proposed by Raij, Azar and Keane [22] and used by Yoshida, Fogo and Ichikawa [23].

Electron microscopy studies were performed in a subset of the whole population; control and treated rats of each strain were selected as reported:

Experimental population (58 animals; C = control, T = treated) used for ultrastructural studies divided by strain and age:

MNS: 1 month (3C); 2 months (2T, 1C); 8 months (6T, 5C); 14 months (5T, 6C).

MHS: 1 month (3C); 2 months (2T, 1C); 8 months (6T, 6C); 14 months (6T, 6C).

Tissue blocks, 1 to 2 mm thick, obtained from the renal cortex were immersed for two hours in 2.5% glutaraldehyde, 0.13 M phosphate buffer pH 7.2, washed in buffer, post-fixed in OsO_4, ethanol dehydrated and embedded in Epon 812.

For each animal, three to five semi-thin sections, obtained from three different blocks, were collected onto a histologic slide and stained with 1% toluidine blue.

From each slide four to five glomeruli were selected under light microscopy, among those without apparent glomerulosclerosis. Accordingly, ultra-thin sections, including the selected glomeruli, were cut, uranylacetate and lead citrate stained and observed with an electron microscope (ElmiSkop 1A, Siemens, Berlin, FRG). Interstitial flogosis was graded from 0 (absent) to 3+ (severe).

Statistical analysis

Results are reported as mean ± SEM, except glomerular TXB_2 levels that are mean ± SD. Data were analyzed by analysis of variance using Duncan's test for multiple comparison or by unpaired Student's *t*-test for single comparisons [24].

The correlation between glomerular TXA_2 production and proteinuria was evaluated using a polinomial regression model [25].

Results

Systolic blood pressure and urine measurements

Values of SBP in control and treated MNS rats are reported in Table 1. SBP rose significantly from four to six weeks of age in both control and FCE 22178-treated animals, and in both groups remained in the range of age-matched normotensive rats of the Okamoto strain (WKY) throughout the study. In fact the pattern of SBP in MNS rats was indistinguishable from that in WKY rats (Fig. 1). Similarly, in MHS rats, treatment with the TX-synthase inhibitor did not alter the time course of hypertension development, nor did it reduce blood pressure in adult hypertensive animals to any statistically significant extent (data not shown). Consistently with the lack of a primary antihypertensive effect of FCE 22178, the drug did not affect the pattern of development of hypertension in SHR rats (Fig. 1).

Data on body weight, water intake and urine volume of MNS and MHS rats are given in Table 2. There were no statistically significant differences in the initial body weight of rats of the two strains, nor in their weight gain up to eight months of age, when body weight of MHS was significantly ($P < 0.05$) higher than that of MNS by approximately 20%.

FCE 22178-treated animals showed slightly lower body weights at 8 and 14 months compared to the corresponding controls.

Young (1 month old) MHS rats had a significantly higher urine volume with respect to matched MNS, but this difference disappeared when hypertension developed, as previously reported [1]. On the other hand, 14-month-old control MNS showed a markedly increased urine volume as compared to matched MHS (41.7 ± 5.0 vs. 11.4 ± 0.8 ml · 24 hr^{-1}; $P < 0.01$).

MNS water intake was increased in parallel. MNS rats receiving FCE 22178 had significantly lower water intake and urinary volumes than MNS receiving vehicle alone, at all experimental time points. The reduced urine volume might be

Table 1. Systolic blood pressure (SBP) of control and FCE 22178-treated rats of the Milan normotensive strain (MNS) at different ages

Age	*N*	SBP *mm Hg* MNS control	SBP *mm Hg* MNS treated
4 weeks	21	121.4 ± 2.4	118.8 ± 3.1
6 weeks	18	135.5 ± 1.9	133.8 ± 2.1
2 months	18	134.1 ± 1.7	133.0 ± 2.2
4 months	12	137.9 ± 2.9	143.7 ± 2.1
6 months	12	142.1 ± 1.6	139.1 ± 3.0
8 months	12	137.9 ± 3.0	145.8 ± 2.2
12 months	6	144.1 ± 2.1	138.3 ± 6.2
14 months	6	150.0 ± 3.1	150.1 ± 3.2

Data are mean ± SEM; $P > 0.05$ for all comparisons control vs. treated.

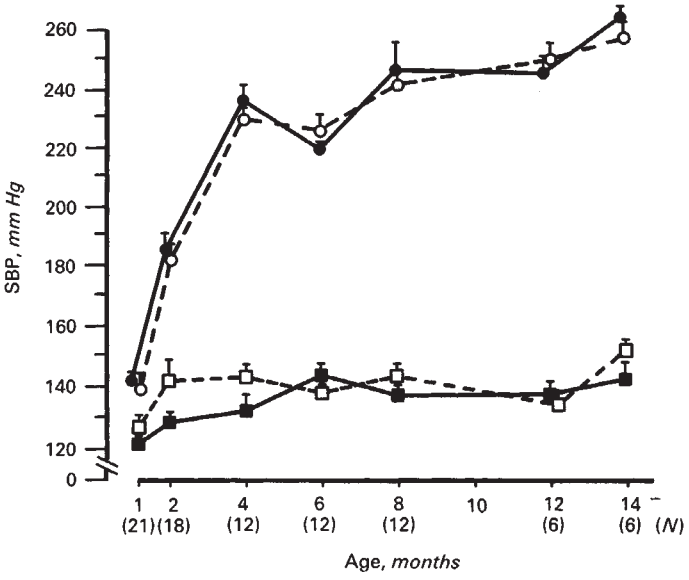

Fig. 1. *Age dependent changes of systolic blood pressure (SBP) measured by tail cuff method in conscious, SHR (control ●, treated ○); and WKY (control ■, treated □) rats.* Treatment with FCE 22178 was performed as reported in the methods. Means ± SEM are reported. The number in parentheses represents the number of animals for each group at different ages.

Table 2. Body weight, water intake and urine volume of control (C) and FCE 22178-treated (T) rats of the Milan normotensive strain (MNS) and of the Milan hypertensive strain (MHS) at different ages

Parameter		1 Month		2 Months		8 Months		14 Months	
		MNS (N = 21)	MHS (N = 21)	MNS (N = 18)	MHS (N = 18)	MNS (N = 12)	MHS (N = 12)	MNS (N = 6)	MHS (N = 6)
Body wt *g*	C	101.5 ± 3.3	96.2 ± 1.7	261.0 ± 4.2	268.5 ± 5.0	432.2 ± 6.9[a]	515.3 ± 9.8	465.2 ± 10.5[a]	549.2 ± 19.9
	T	105.3 ± 3.0	109.0 ± 2.0	256.2 ± 4.1	264.8 ± 4.9	418.3 ± 6.7	487.1 ± 14.6	433.0 ± 6.1	504.8 ± 13.8
H_2O intake *ml/24 hr*	C	17.1 ± 0.6	18.0 ± 0.7	24.1 ± 0.8 [c]	24.8 ± 0.7	23.7 ± 1.3 [d]	24.4 ± 1.2	47.1 ± 4.0 [d]	24.6 ± 1.0
	T	17.9 ± 0.8	20.7 ± 0.8	21.5 ± 0.5	24.6 ± 0.6	17.8 ± 1.1	21.3 ± 1.1	28.1 ± 5.0	22.7 ± 1.0
Urine volume *ml/24 hr*	C	4.01 ± 0.1[a]	5.90 ± 0.4	8.43 ± 0.3 [c]	8.82 ± 0.4	14.1 ± 0.7 [d]	13.5 ± 0.7	41.7 ± 5.0[b] [d]	11.4 ± 0.8
	T	4.69 ± 0.1	6.50 ± 0.3	6.81 ± 0.2	8.82 ± 0.3	10.2 ± 0.4	12.0 ± 0.7	18.8 ± 4.1	12.8 ± 0.7

Data are means ± SEM.
[a] $P < 0.05$, [b] $P < 0.01$ control MNS vs. control MHS
[c] $P < 0.05$, [d] $P < 0.01$ control vs. treated by Duncan's test

Table 3. Urine protein excretion in control rats of the Milan normotensive strain (MNS) and of the Milan hypertensive strain (MHS) as a function of aging

Age	N	Urine protein *mg · 24 hr*$^{-1}$ MNS	MHS
4 weeks	21	3.5 ± 0.4[a]	1.9 ± 0.3
6 weeks	18	21.9 ± 1.9[b]	9.1 ± 0.7
2 months	18	40.1 ± 1.6[a]	32.1 ± 1.7
4 months	12	122.8 ± 4.2[b]	38.3 ± 4.7
6 months	12	288.3 ± 30.6[b]	22.2 ± 1.8
8 months	12	385.2 ± 37.7[b]	24.5 ± 1.8
12 months	6	471.9 ± 51.4[b]	22.1 ± 3.5
14 months	6	625.3 ± 94.1[b]	24.2 ± 2.5

Data are means ± SEM.
[a] $P < 0.05$; [b] $P < 0.01$ MNS vs. MHS by Duncan's test

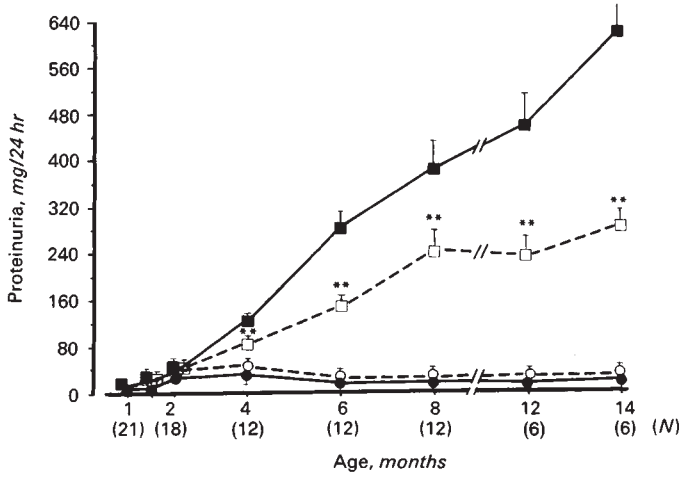

Fig. 2. *Twenty-four-hour protein excretion of MNS (control ■, treated □) and MHS (control ●, treated ○) at different ages.* Treatment with FCE 22178 was performed as described in the methods. Means ± SEM are reported; the number in parentheses represents the number of animals for each group at different ages. ** $P < 0.01$ by Duncan's test in comparison to age matched controls.

due to an improvement in concentrating capability in treated MNS as a consequence of a lower degree of tubular atrophy as suggested by histologic findings (see below). Treatment did not modify these parameters in MHS. Food intake was normal in both control and treated rats of the two strains. In addition, we did not observe any abnormal behavior, nor diarrhea.

Total urinary protein excretion of control MNS and MHS rats from 1 to 14 months of age, is detailed in Table 3. In MNS marked proteinuria developed after the fourth month, becoming massive by 14 months of age (625.3 ± 94.1 mg · 24 hr^{-1}); however, even young MNS had a significantly higher protein excretion than MHS rats at the beginning of the experiment (3.5 ± 0.4 vs. 1.9 ± 0.3 mg · 24 hr^{-1}, $P < 0.05$). On the other hand, unlike other hypertensive strains, proteinuria did not develop in old hypertensive MHS (24.2 ± 2.5 mg · 24 hr^{-1}, at 14 months of age).

The high urinary protein excretion of 14-month-old MNS was paralleled by a lower plasma concentration of albumin (16.8 ± 2.3 mg · ml^{-1}, N = 5) as compared to normal values in normotensive rats of that age (mean value 35.0 ± 0.08 mg · ml^{-1}) as shown by Berg [26]. Treatment with the TX-synthase inhibitor reduced, but did not abolish, the proteinuria of MNS rats. Total protein excretion (expressed in mg · 24 hr^{-1}) of treated MNS was significantly lower than that of control MNS after the fourth month of age (Fig. 2). The extent of reduction was remarkably constant throughout the study, ranging from 50% at 6 months to 53% at 14 months of age (291.7 ± 55.4 in treated vs. 625.3 ± 94.1 mg · 24 hr^{-1} in control rats at 14 months of age).

Treatment with the TX-synthase inhibitor was also associated with significantly higher plasma albumin levels, as measured at 14 months (from 16.8 ± 2.3 in control to 25.3 ± 3.2 mg · ml^{-1} in treated MNS, $P < 0.01$, N = 5).

FCE 22178 had no effect on total protein excretion of MHS rats (Fig. 2).

Renal clearance in chronic and acute studies

Figure 3 depicts the age-related changes in renal hemodynamics measured in MNS and MHS rats during the long-term study; in control MNS both RPF (measured as ^{125}I-hippuran clearance) and GFR (measured as ^{51}Cr-EDTA clearance) were significantly decreased at 8 months, and markedly reduced at 14 months of age when compared to basal values: RPF from 4.0 ± 0.2 to 1.7 ± 0.3 ml · min^{-1} · g^{-1} kidney weight ($P < 0.01$); GFR from 1.4 ± 0.06 to 0.5 ± 0.1 ml · min^{-1} · g^{-1} kidney wt, ($P < 0.01$). In contrast, both parameters remained remarkably stable during the entire life span of MHS rats. Therefore both RPF and

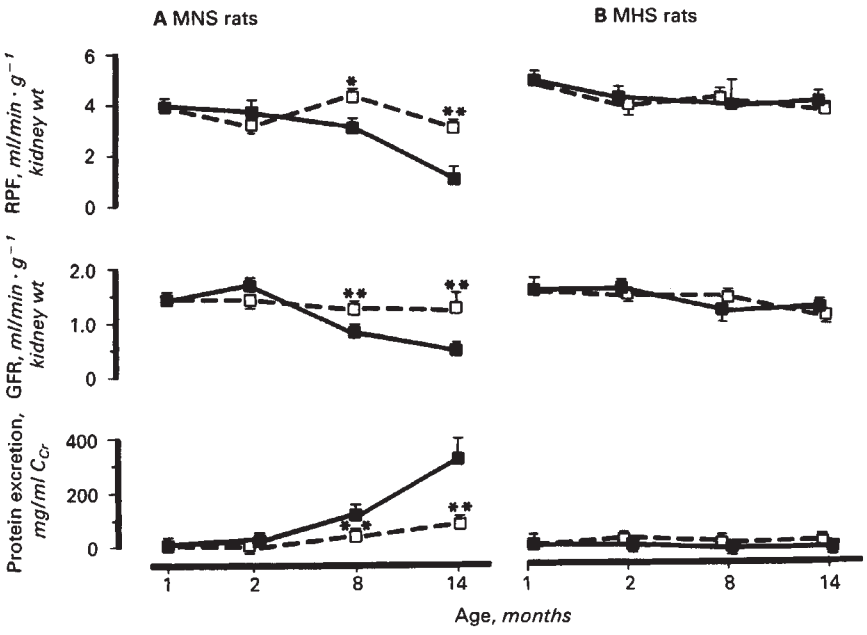

Fig. 3. *Age-related changes of renal plasma flow (RPF), glomerular filtration rate (GFR) and 24-hours urinary protein excretion expressed per ml of ^{51}Cr-EDTA clearance in control (■) and treated (□) MNS (A) and MHS rats (B).* Treatment with FCE 22178 was performed as reported in the methods. Means ± SEM of 5 to 6 animals are shown. * $P < 0.05$; ** $P < 0.01$ by Duncan's test in comparison to age-matched controls.

GFR, which were similar in the two groups at the beginning of the study, became significantly lower in MNS than in MHS, at 8 and 14 months. Treatment with the TX-synthase inhibitor substantially preserved renal function in MNS rats; RPF did not fall with age, remaining significantly ($P < 0.01$) higher than in controls both at 8 and 14 months of age (at 14 months 3.3 ± 0.08 in treated vs. 1.7 ± 0.3 ml · min^{-1} · g^{-1} kidney wt in control MNS). Similarly, FCE 22178 largely prevented the decrease in GFR at the same experimental time points (at 14 months 1.3 ± 0.2 in treated vs. 0.5 ± 0.1 ml · min^{-1} · g^{-1} kidney wt in control MNS, $P < 0.01$). When taking into account these differences in GFR, the effect of the inhibitor on glomerular permeability to proteins appears even more pronounced than reflected in Figure 2. Thus, total protein excretion of the animals undergoing clearance studies (expressed per milliliter of GFR), was reduced by approximately 75% at 14 months in treated MNS with respect to age-matched controls (Fig. 3). In MHS rats, on the contrary, RPF, GFR and total protein excretion were similar in control and treated animals at all experimental time points.

In acute studies, RPF and GFR of 12-month-old control MNS were significantly lower ($P < 0.01$) as compared to those of normal, 3-month-old Sprague-Dawley rats (2.9 ± 0.7 vs. 5.8 ± 1.0 and 0.2 ± 0.06 vs. 1.6 ± 0.3 ml · min^{-1} · g^{-1} kidney wt, respectively). Saline infusion had no effect on these parameters in MNS. Thromboxane synthesis inhibition with FCE 22178 and/or thromboxane receptor antagonism with BM 13,177 failed to modify acutely RPF and GFR in MNS rats (Table 4). Proteinuria was also similar in control and treated MNS both under basal conditions and four hours after saline or drug infusion (data not shown).

Measurement of serum lipids

Serum cholesterol, phospholipids and triglycerides were not significantly different in one-month-old MHS and MNS rats and were in the normal range for rats of that age [27] (Fig. 4).

No age-related increase in serum lipids was detected in control MHS rats. On the other hand, serum lipids of MNS showed a progressive increase with age and reached very high levels at 14 months: cholesterol (218.0 ± 30.0 mg · dl^{-1}), phospholipids (256.8 ± 26.4 mg · dl^{-1}) and triglycerides (399.6 ± 104.7 mg · dl^{-1}) were all significantly ($P < 0.01$ by unpaired t-test) elevated when compared to one month values as well as to measurements performed in age-matched MHS rats.

FCE 22178 had no effect on serum lipids of MHS, but largely prevented the age-related increase in cholesterol, phospholipids and triglycerides of MNS rats (117.8 ± 3.9; 160.2 ± 15.1 and 169.3 ± 26.1 mg · dl^{-1}, respectively, at 14 months).

Table 4. Renal hemodynamic parameters in 12-month-old rats of the Milan normotensive strain (MNS) treated with saline (0.5 ml · h^{-1}), FCE 22178 (10 mg · kg^{-1} · h^{-1}), BM 13,177 (3 mg · kg^{-1} · hr^{-1}) or both

Group	N	C_{PAH} ml · min^{-1} · g^{-1} kidney wt[a]		C_{In} ml · min^{-1} · g^{-1} kidney wt[b]	
		Control period	Drug infusion (4th hr)	Control period	Drug infusion (4th hr)
Normal rats	8	5.8 ± 1.0	—	1.6 ± 0.3	—
MNS					
Saline	5	2.9 ± 0.7	2.3 ± 0.6	0.19 ± 0.06	0.32 ± 0.09
FCE 22178	9	2.1 ± 0.5	3.7 ± 1.3	0.20 ± 0.06	0.35 ± 0.13
BM 13177	6	2.7 ± 0.7	2.7 ± 0.5	0.17 ± 0.04	0.18 ± 0.07
BM 13177 + FCE 22178	6	2.6 ± 0.5	2.6 ± 0.6	0.27 ± 0.05	0.36 ± 0.1

Data are means ± SEM.
[a] p-aminohippuric acid clearance
[b] inulin clearance

Glomerular TXA_2 synthesis

Figure 5 depicts the ex vivo glomerular production of TXA_2, (measured as TXB_2, 6 hours after the last treatment) in control and FCE 22178-treated MNS and MHS rats at 1, 2, and 14

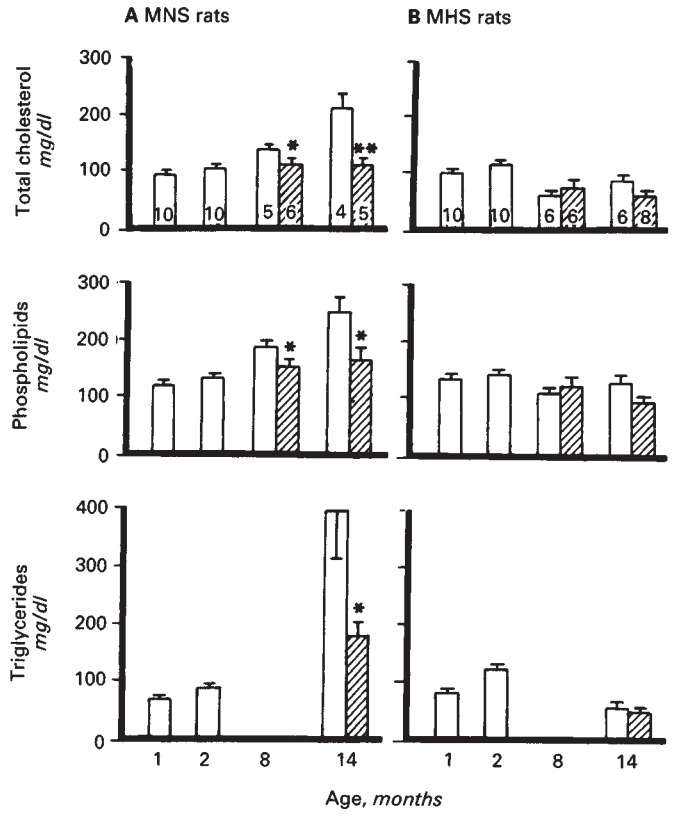

Fig. 4. *Total serum cholesterol, phospholipids and triglycerides in control (□) and treated (▨) MNS and MHS rats at different ages.* Treatment with FCE 22178 was performed as reported in the methods. Means ± SEM of (*N*) animals are shown. * $P < 0.05$; ** $P < 0.01$ in comparison to age matched saline-treated control rats by unpaired *t*-test. Number of animals of each group is reported in the 1st series of bars.

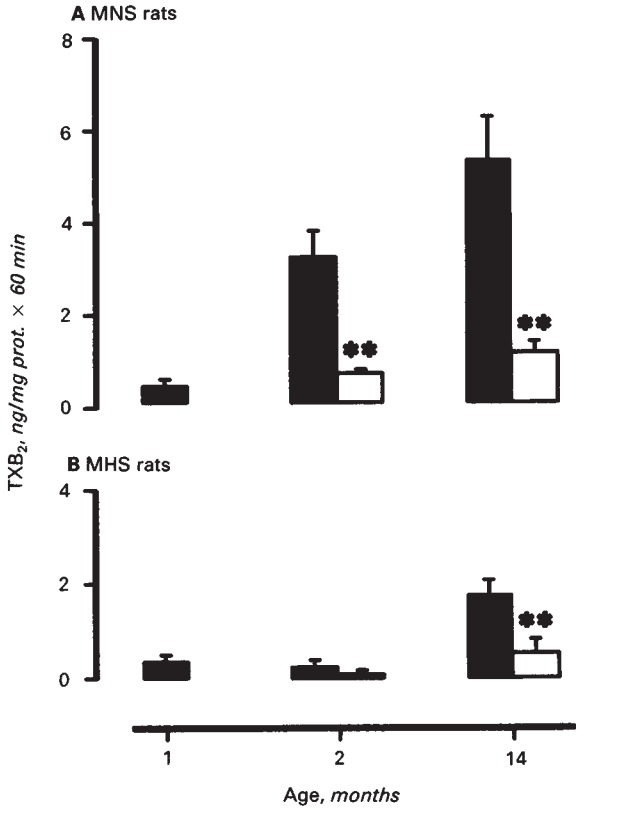

Fig. 5. *TXB_2 production by isolated glomeruli (N = 6) from control (■) and treated (□) MNS and MHS rats at different ages.* Treatment with FCE 22178 was performed as reported in the methods. Data are shown as means ± SD; ** $P < 0.01$ by unpaired *t*-test in comparison to age matched controls.

months of age. Other arachidonate metabolites were not measured as data on the glomerular synthesis of PGE_2 and PGI_2, at different ages, were already available from previous studies in both strains [28].

As early as the second month of life, glomerular synthesis of TXA_2 was increased several-fold in MNS rats. A 12-fold increment was measured at 14 months (from 0.47 ± 0.1 at 1 month to 5.7 ± 0.9 ng · mg^{-1} prot · hr^{-1}, $P < 0.01$ by unpaired *t*-test).

Glomerular TXB_2 production increased significantly also in MHS though only at 14 months of age and to a lesser extent than in MNS (from 0.49 ± 0.1 at 1 month to 1.8 ± 0.2 ng · mg^{-1} prot · $hour^{-1}$, at 14 months, $P < 0.01$).

MNS rats receiving the TX-synthase inhibitor showed markedly reduced glomerular TXB_2 production (80%) at both experimental time points. A statistically significant reduction in glomerular TXB_2 production was also observed in treated MHS rats at 14 months.

To assess the relationship between ex vivo glomerular TXA_2 synthesis and glomerular injury, TXB_2 levels were correlated with urinary protein excretion and GFR in both control and treated MNS. A statistically significant correlation ($r = 0.72$, $N = 39$, $P < 0.01$) was found between glomerular TXB_2 production and urinary protein excretion (Fig. 6). Moreover, an

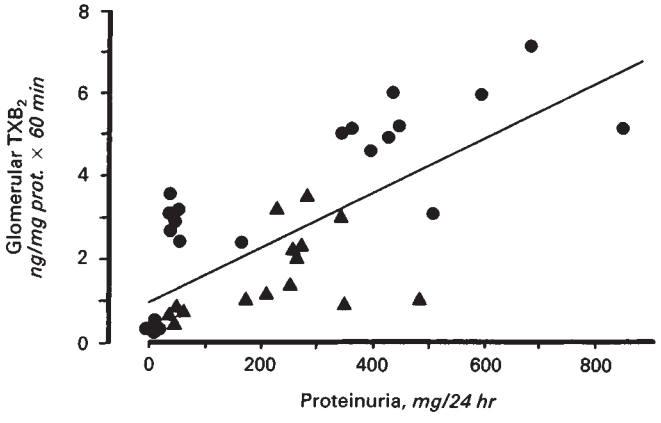

Fig. 6. *Correlation between the presacrifice 24-hours urine protein excretion (proteinuria · 24 h^{-1}) and the post-sacrifice ex vivo glomerular TXB_2 synthetic rate in MNS rats at different ages.* Control (●) and treated (▲) rats are plotted together. Treatment with FCE 22178 was performed as reported in the methods. $N = 39$; $r = 0.72$; $P < 0.01$.

inverse correlation ($r = -0.63$, $N = 39$, $P < 0.01$) was found between TXB_2 synthetic rate and GFR (^{51}Cr-EDTA clearance; not shown).

Table 5. Percentage sclerotic glomerular area in rats of the Milan normotensive strain (MNS) and in rats of the Milan hypertensive strain (MHS) at 8 and 14 months of age

Age *months*	MNS		MHS	
	Control	Treated	Control	Treated
8	30.5 ± 3.1[b] (126)	17.5 ± 1.6[a] (148)	4.3 ± 0.9 (132)	4.6 ± 0.8 (159)
14	46.1 ± 3.9[b] (140)	34.2 ± 4.7[a] (149)	8.4 ± 1.6 (165)	9.9 ± 1.1 (190)

The mean number of glomeruli measured in each kidney are reported in parentheses. Data are means ± SEM (N = 5 to 6 kidneys per group). The % sclerotic area was calculated on the basis of a sclerotic index as reported in **Methods**.

[a] $P < 0.01$ vs. corresponding control (MNS) values

[b] $P < 0.01$ vs. corresponding control MHS

Morphology

Light microscopy. No appreciable histologic abnormalities were observed in one and two month old treated as well as untreated MNS. Lesions observed in 8- and 14-month old animals included focal glomerular sclerosis and hyalinosis, with a segmental or global pattern. The capillary lumina were often narrowed or even obliterated by PAS and PASM+ material, that was preferentially located in the mesangial compartment. Tubules showed atrophy of epithelial cells with occasional proteinaceous casts in the lumen. Percentage sclerotic area, calculated from the sclerosis index as reported in the methods, is shown in Table 5.

MNS had a significantly higher ($P < 0.01$) percent sclerotic area at 8 months of age (range 22 to 37%; $N = 6$) as compared to MHS (range 2 to 8%; $N = 6$). This difference was more marked at 14 months (range 37 to 61%; $N = 6$ vs. 4 to 14%; $N = 6$). Treatment with FCE 22178 significantly ($P < 0.01$) reduced the percent of sclerotic area in MNS at both experimental time points, but was without any measurable effect in MHS. At variance with MNS, 8-month-old MHS showed a moderate thickening of the media of the interlobular arteries which was slightly more pronounced at 14 months. This parameter was not modified by treatment.

Electron microscopy. In the present preliminary study the ultrastructural investigation has been mainly focused on the glomerular pattern of control rats of the two strains, and differences between treated and untreated animals were more qualitative than quantitative.

At 28 days, which was assumed as the basal condition, the glomerular structure of MNS rats did not show any significant abnormality. At two months of age, the glomerular pattern was unmodified in both treated and untreated animals, with only occasional focal areas of homogeneous thickening of the lamina densa (LD) of the glomerular basement membrane (GBM). At 8 and 14 months of age, the ultrastructure of the glomerular components showed appreciable abnormalities definitely more pronounced in older animals. A frequent finding observed in almost all glomeruli of untreated animals was a generalized flattening and fusion of foot processes, with consequent collapse of the epithelial cell body over the GBM (Fig. 7). On the contrary, in the glomeruli of FCE 22178-treated animals, epithelial cells showed only moderate aspects of foot process fusion and in some cases the morphology was considerably well preserved (Fig. 8).

Other common findings more pronounced and frequent in untreated than in treated animals were: a) villosity with production of abundant irregular cytoplasmatic projections protruding into the capsular space (Fig. 7 inset); b) increased number of particularly electron-dense lysosomes and presence of large vacuoles and wide electronlucent blebs, surrounded by a thin rim of cytoplasm (Fig. 7); and c) presence of considerable amounts of filamentous material, morphologically consistent with actin, often arranged at the periphery of the cytoplasm. In most severe cases, focal aspects of the detachment of podocytes from the GBM were observed (Fig. 9). The latter finding was absolutely exceptional in treated MNS.

Finally, the mesangial matrix appeared markedly increased as a consequence of the deposition of basement membrane-like material, which often caused obliteration of the capillary lumen. This feature was definitely less frequent and less pronounced in FCE 22178-treated animals.

No appreciable presence of platelets was detected in any of the examined glomeruli. In MNS rats interstitial flogosis was modest (1+) at 8 months and slightly higher (1+/2+) at 14 months; the degree of flogosis was not modified by treatment with FCE 22178.

Discussion

The results of the present study show that the renal disease of MNS rats is characterized by persistent proteinuria, hypoalbuminemia and hyperlipidemia, and a progressive impairment of renal function. An interesting finding was that arterial blood pressure remained at normotensive levels throughout the MNS life span, unlike other models of renal damage with similar pathologic features [29, 30]. This observation would tend to minimize any role for systemic hypertension in the development of glomerular damage in MNS. In this series of experiments we did not measure glomerular capillary pressure (P_{gc}) and therefore we cannot exclude glomerular hypertension in the late stages of the disease. However, previous studies showed that in younger animals (3 months of age) P_{gc} was significantly ($P < 0.01$) lower in MNS as compared to MHS rats [31].

We found sclerotic glomerular changes after the eighth month of age. Ultrastructural studies of nonsclerotic glomeruli showed thickening of the GBM and an age related increase of mesangial matrix which appeared to be strictly related to the structural modifications of epithelial cells. Flattening and fusion of the foot processes, as well as formation of blebs and of electron-dense lysosomes, together with the frequent detachment of the cells from the GBM might be related to the massive leakage of proteins through the glomerulus [32].

These data confirm and extend previous observations of Brandis et al [4], who first described glomerulosclerosis and proteinuria in MNS rats. The absence of rat complement and IgG in the glomeruli led the authors to suggest that an immunologic mechanism is unlikely to be responsible for this glomerulopathy. We also extended preliminary observations of an age-related increase in glomerular synthesis of TXA_2, as detected ex vivo by the production of the stable hydrolysis product, TXB_2 [28]. Other experimental models of non-immune as well as immune renal damage show abnormalities in the intrarenal metabolism of arachidonate [7–10, 33–35]. However,

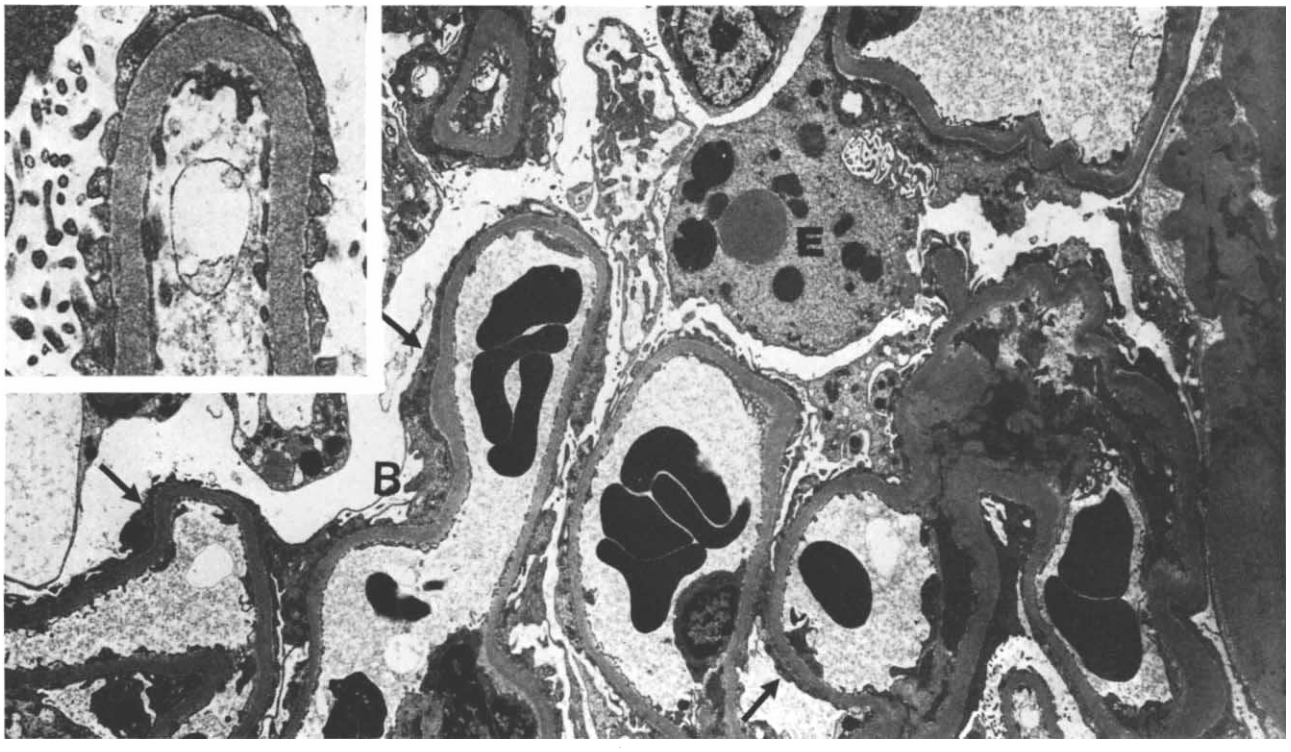

Fig. 7. *Untreated MNS rat, 14 month old: (2200×).* Glomerular epithelial cells (E) show electrondense lysosomes, a bleb (B), flattening and fusion of foot processes (arrows). *INSET*: high magnification of the same picture showing villosity of an epithelial cell.

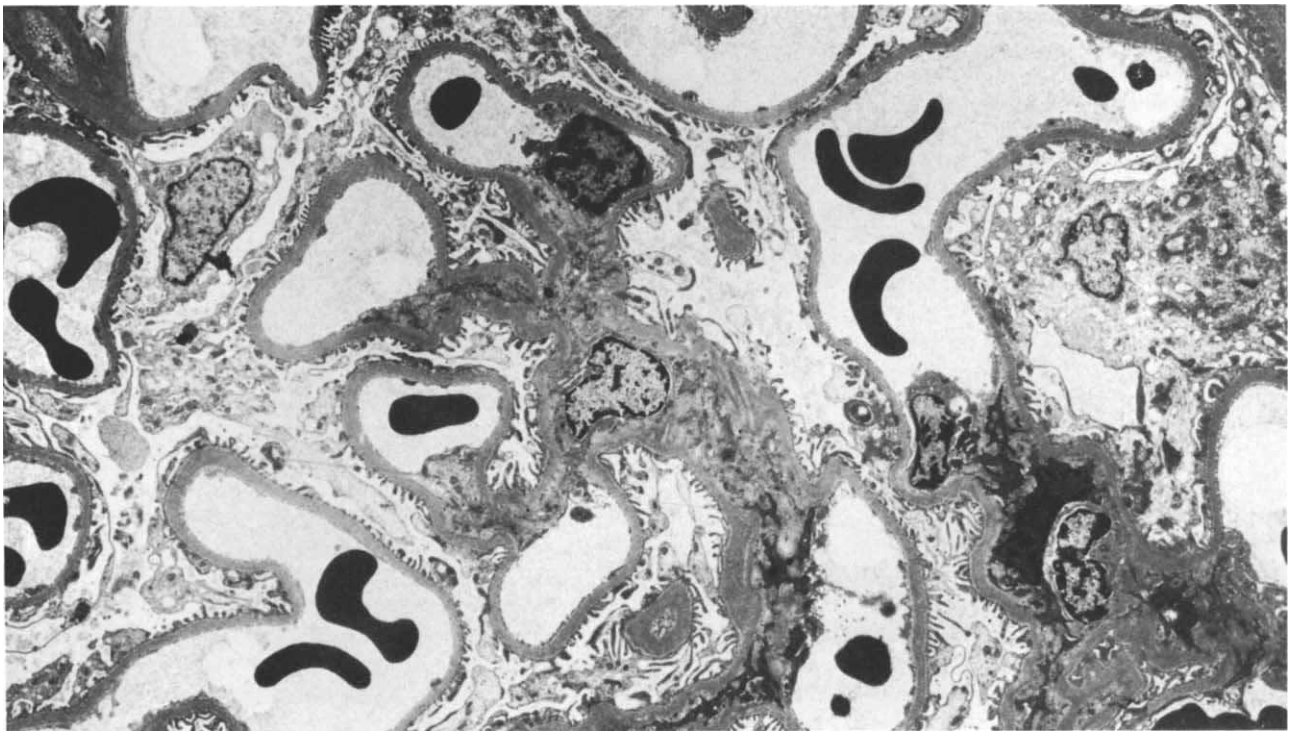

Fig. 8. *Treated MNS rat, 14 month old: (2200×).* Note the preservation of the foot processes compared to Fig. 7.

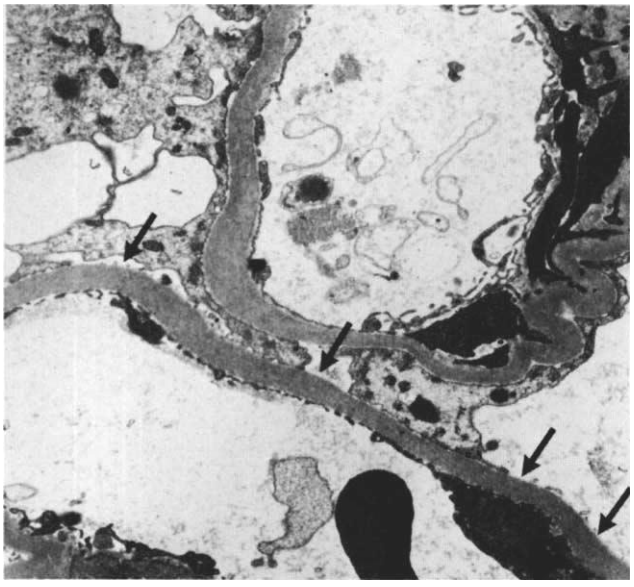

Fig. 9. *Untreated MNS rat, 14 month old: (5800×).* Arrows point to a focal detachment of the epithelial cell surface from the GBM.

if we exclude examples of autoimmune kidney disease, such as the murine model of lupus nephritis [36], the MNS glomerulosclerosis is the first example, to our knowledge, of a spontaneous, non-immune, genetically determined renal disease in the rat, characterized by a slow onset and long lasting proteinuria, where a role for intraglomerular TXA_2 production has been established on the basis of biochemical measurements and inhibitor trials. In this study, in fact, we found that the oral administration of FCE 22178, an imidazole-analogue inhibitor of TXA_2-synthase, slowed the progression of MNS renal disease. Protein excretion was markedly decreased by FCE 22178; the reduction of proteinuria did not appear to be a consequence of a reduction in tubular load as GFR was not decreased by the drug. On the contrary, GFR and RPF were significantly higher in treated animals than controls, at both 8 and 14 months of age, and were not significantly different from baseline values. On the basis of these data, it is likely that FCE 22178 did not decrease P_{gc} and actually increased glomerular perfusion and filtration in this animal model; nevertheless the percent of sclerotic glomerular area was less in treated as compared to control rats. These data are consistent with the findings of Purkerson et al, who have shown that a TXA_2-synthase inhibitor (OKY 1581) ameliorates the progressive kidney disease in the rat model of reduced renal mass in the face of a further increase in glomerular hyperfiltration [10]. However, in that study, treatment with OKY 1581 was also associated with a fall in systemic blood pressure, an effect likely contributing to the improvement in renal histology.

In our study, the beneficial effects of FCE 22178 on renal function and structural integrity were not associated with any significant change in systemic blood pressure, thereby implying a largely intrarenal mechanism of action. As we did not measure glomerular hemodynamics, one might speculate that FCE 22178 normalized intraglomerular capillary pressure, possibly increased in old MNS. Alternatively, MNS might behave like puromycin-treated rats, in which P_{gc} remains normal in spite of reduced renal function [37]. In that model, TXA_2-synthase inhibitors have been shown to exhert beneficial effects on renal function and proteinuria without modifying P_{gc} [38, 39].

Moreover, our results in control MNS are at variance with the findings in rats with subtotal nephrectomy in that a lower degree of glomerular sclerosis was found. This difference might reflect the additional glomerular damage induced by systemic hypertension in the reduced renal mass model. On the other hand, Yoshioka et al [40] have recently shown that in the subtotal nephrectomy model, nephrons without glomerulosclerosis (that is, with intact structure at light microscopy) often had the highest filtration rate of albumin. Thus, proteinuria after subtotal nephrectomy originates largely from glomeruli with minimal structural abnormalities. These observations have raised the possibility that in chronic renal diseases, the reduction in proteinuria seen after a variety of therapeutic measures, including TX-synthase inhibition, may reflect their functional effect on the relatively intact glomeruli rather than their structure-sparing effect on severely damaged glomeruli, which contribute little to the proteinuria [40]. Our present results are consistent with such a possibility, in showing up to 75% reduction in proteinuria in the face of only a 26% sparing in sclerotic area.

Our results do not address the questions as to the cellular origin of enhanced glomerular TXB_2 production and to the molecular transduction of this biochemical abnormality into changes of glomerular function and structure. However, interstitial flogosis was also modest in the histologic sections from old animals (14 months), whereas enhanced glomerular production of TXB_2 was also present in younger rats. No platelet deposition could be demonstrated.

These observations point to native glomerular cells as the main source of TXA_2. Yet, we cannot rule out a contribution to increased glomerular TXA_2 by infiltrating cells in a late phase of the renal disease. Therefore a possible role of intraglomerular TXA_2-dependent platelet activation in this particular disease process, as suggested by Purkerson et al [10, 41] in the remnant kidney model. remains to be investigated with appropriate pharmacologic tools.

Moreover, the genetic determinant(s) responsible for the age-related increase in TXA_2 synthesis remains to be defined.

Because enhanced protein excretion preceded any detectable increase in glomerular TXB_2 production and profound suppression of the latter did not completely prevent development of the disease, we are inclined to suggest that TXA_2 represents an important mediator amplifying glomerular injury in response to a variety of mechanisms [42–44] and not a primary determinant of the disease. Preservation of RBF and GFR in animals treated with FCE 22178 is consistent with the effects of similar inhibitors of TX-synthase in other rat models [7, 9, 13] and suggests important effects of locally generated TXA_2 on glomerular arterioles and mesangial cells [12]. Our study involved long term treatment with a TX-synthase inhibitor and initiated at an early stage of the disease; the question whether similar beneficial effects on renal hemodynamics can be obtained at a late stage, in the face of a full blown picture of kidney disease, has been partially addressed in our acute study in 12-month-old MNS. Four hour infusion of a dose of FCE 22178, completely inhibiting TXB_2 formation in whole blood during clotting, did

not significantly modify RPF or GFR. A vasoconstrictive activity of endoperoxides accumulating in presence of TX-synthase inhibition can be excluded by the experiments performed treating the rats with both FCE 22178 and the PGH_2/TXA_2 receptor antagonist, BM 13,177.

Therefore, neither TXA_2, nor vasodilator prostaglandin (PG) synthesis (possibly increased by PGH_2 rediversion) seem to acutely modulate renal hemodynamics in already diseased MNS. Other experimental models, for example, streptozotocin diabetic rats, characterized by enhanced glomerular production of TXA_2 and PGs [45] associated with hyperfiltration and proteinuria [46], have recently been reported to get benefit from TX-synthase inhibition, irrespective of changes in renal hemodynamics [47]. We suggest that TX-synthase inhibition might ameliorate renal function in chronic studies also by playing a modulating role on mesangial cell growth. TXA_2 and PGs in fact have been implicated in the regulation of the growth of many cell types including glomerular cells [48, 49]. It could be speculated that an altered balance between growth-stimulating and growth-inhibitory cyclooxygenated products may contribute, at least partially, to the development and progression of glomerular lesions in certain experimental models of proteinuria.

Previous work by one of us has shown that in MNS rats, in addition to TXA_2, glomerular production of prostaglandin $(PG)E_2$ and PGI_2 (prostacyclin) is also increased [28].

Finally, the hyperlipidemia which was evident from the eighth month of life in control MNS, was dramatically reduced by FCE 22178. A direct action of the drug on lipid metabolism can be ruled out on the basis of indirect evidence obtained in a different rat model of experimental hyperlipidemia where chronic treatment with FCE 22178 did not modify serum total cholesterol (unpublished observation). On the other hand, a complex disorder of plasma lipoproteins with hypertriglyceridemia and hypercholesterolemia similar to that found by us in MNS rats has been described in rats with nephrotic syndrome induced by puromycin aminonucleoside [50] or by adriamycin [20], and also in rats with subtotal nephrectomy [51].

These data, taken together, suggest that the nephrotic syndrome itself might be the main cause of hyperlipidemia in MNS rats. Therefore the beneficial effect of FCE 22178 on the lipid disorder is likely secondary to the normalization of renal function and the reduction of proteinuria. In conclusion, our present results indicate that, although many features of the disease are similar to those of other models of renal damage in the rat, the MNS glomerulosclerosis is not associated with systemic hypertension. On the other hand, MNS rats represent the first, non-immune, genetic model of long lasting proteinuria and age-related glomerulosclerosis in which enhanced intrarenal synthesis of TXA_2 has been documented and correlated with functional and structural abnormalities.

The beneficial effects obtained with FCE 22178 suggest that TX-synthase inhibitors might open new perspectives in the therapy of chronic renal disease and associated lipid disorders.

Acknowledgments

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