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# Acute and chronic effects of thromboxane A<sub>2</sub> inhibition on the renal hemodynamics in streptozotocin-induced diabetic rats

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**Acute and chronic effects of thromboxane A<sub>2</sub> inhibition on the renal hemodynamics in streptozotocin-induced diabetic rats.** We examined acute and chronic effects of thromboxane (TX) A<sub>2</sub> inhibition on the renal hemodynamics at early and late stage of untreated streptozotocin (STZ)-induced diabetic rats. Two weeks and 28 weeks after the induction of diabetes, renal blood flow (RBF) under anesthesia was measured with an electromagnetic flowmeter before and after TXA<sub>2</sub> inhibition. In two-week-old diabetic rats, a specific TXA<sub>2</sub> synthetase inhibitor, OKY-046, or a specific TXA<sub>2</sub> receptor antagonist, Sulotroban, increased renal vascular resistance (RVR) and ameliorated the hyperperfusion. The renal vasoconstrictive effect of OKY-046 was blunted by an angiotensin converting enzyme (ACE) inhibitor, MK422, or an angiotensin II receptor antagonist, Saralasin. On the contrary, OKY-046 ameliorated the renal hypoperfusion by decreasing RVR in 28-week-old diabetic rats. Chronic oral administration of OKY-046 ameliorated not only the renal hyperperfusion but increased urinary albumin excretion (UAE) at two weeks, but also the renal hypoperfusion, filtration fraction and UAE at 24 weeks. It is suggested that TXA<sub>2</sub> might, at least in part, play important roles in the hyperperfusion by modulating activity of the renin-angiotensin system at an early stage of untreated diabetic rats and in the hypoperfusion at the late stage of untreated diabetic rats, and that TXA<sub>2</sub> is also involved in the increase of UAE. These results support roles for TXA<sub>2</sub> in the progression of renal injury in STZ-induced diabetic rats.

It is well known that renal blood flow (RBF) and glomerular filtration rate (GFR) increase in patients with newly diagnosed insulin-dependent diabetes mellitus (IDDM) and in diabetic rats with moderate hyperglycemia or with short-term severe hyperglycemia [1, 2]. The increases in RBF and GFR are considered to be important factors in the development and the progression of diabetic nephropathy [1, 2]. The mechanisms of hyperperfusion and hyperfiltration, however, are still unknown. The roles of prostaglandins (PGs) in the renal hemodynamic changes of diabetes have been extensively studied [3–9]. Synthesis of PGE<sub>2</sub> and PGI<sub>2</sub> in isolated glomeruli from diabetic rats with moderate hyperglycemia and with short-term severe hyperglycemia is increased [3–6]. Administrations of non-steroidal anti-inflammatory drugs ameliorate the renal hyperperfusion and the glomerular hyperfiltration in IDDM patients [7] and in diabetic rats [8, 9]. These data indicate that vasodilatory PGs are

involved in renal and glomerular hemodynamic abnormalities in diabetic subjects [1, 2, 10]. The role of thromboxane (TX) A<sub>2</sub> in diabetic nephropathy has also been investigated [3, 6, 11–16]. Urinary excretion of TXB<sub>2</sub> increases in diabetic patients [11] and diabetic rats [12–14]. Administration of TXA<sub>2</sub> synthetase inhibitors ameliorates microalbuminuria in IDDM patients [15, 16] and diabetic rats [14]. In diabetic rats with severe hyperglycemia for 2 months, there is enhanced intraglomerular TXA<sub>2</sub> synthesis, resulting in a decreased GFR [6]. Glomerular synthesis of TXA<sub>2</sub> is also increased in four-week-old, severely hyperglycemic diabetic rats, in which an administration of TXA<sub>2</sub> synthetase inhibitor enhances the hyperfiltration [3]. However, the role of TXA<sub>2</sub> in diabetic renal hemodynamics remains unclear. The aim of this study is to investigate the role of TXA<sub>2</sub> on the renal hemodynamics in untreated streptozotocin (STZ)-induced diabetic rats.

## Methods

Male Sprague-Dawley rats (Seiwa, Fukuoka, Japan) weighing 200 to 270 g were made diabetic by intraperitoneal injection of STZ (90 mg/kg). Rats for normal control were given injection of the vehicle alone; diabetic rats received no insulin. They were allowed free access to water and a regular rat pellet diet (Na 0.12 mEq/g). Diabetic rats in which non-fasting blood glucose levels were more than 300 mg/dl were studied.

The procedures for measuring RBF were as follows [17]. The rats were maintained on a regular diet until 10 to 14 hours before the experiment, at which time the food was removed and the rats were allowed free access to water until the time of anesthesia. The rats were anesthetized with Inactin (80 mg/kg), administered intraperitoneally. Rats were placed on a heat-controlled table to maintain the rectal temperature at 37°C. After tracheostomy was performed, PE-50 catheters were placed in the right jugular vein and the left femoral artery, and a PE-90 catheter was placed in the urinary bladder. Arterial blood pressure was monitored continuously throughout the experiments with a P231B Gould-Statham pressure transducer and recorded on a heat-writing recorder (WT-625G, Nihonkoden, Tokyo, Japan). The left kidney was exposed through abdominal incision, and the left renal artery was freed from the surrounding tissues. The renal nerves were carefully avoided during dissection and manipulation of the renal artery. The arterial branches which did not enter into the kidney were ligated. A flow probe (EP102, size 2 mm circular, Carolina

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Medical Electronics, King, North Carolina, USA) was placed on the left renal artery. The probe was fixed on a flexible arm, by which the position of the probe on the renal artery could be stabilized. The abdomen was not closed around the probe and the positioning of the probe was observed under microscopy throughout the experiments. The flow probe was connected to the flowmeter (Model FM501, Carolina Medical Electronics). RBF was continuously monitored throughout the experiments and recorded on the heat-writing recorder. Sixty minutes were allowed after the surgery before any experimental procedures or measurements were made. At the end of the experiments, the zero flow line of RBF was verified by occlusion of the renal artery distal to the probe. To replace the loss of fluid associated with surgical preparations, 1% of body weight of rat plasma was infused over 60 minutes, then followed by an infusion of rat plasma at the rate of 0.2% of body wt/hr throughout the experiments. Rat plasma obtained from normal rats was infused into normal rats, and that obtained from diabetic rats was infused into diabetic rats. Rats were also maintained on an infusion of physiological saline at the rate of 2 ml/hr during surgical procedures, and at the rate of 1 ml/hr during the experimental protocols. Preliminary study revealed that this protocol maintained constant systemic hematocrit and arterial pressure. RBF was measured every ten minutes during a basal period of 30 minutes (control period), and during a subsequent experimental period of 30 minutes (experimental period) that commenced 30 minutes after starting an intravenous infusion of the drugs. The coefficient of variation of the three measurements of RBF during each period in normal control rats was 3.7%. RBF in normal control rats was stable for 120 minutes after the 60 minute equilibration period ( $N = 5$ ,  $9.3 \pm 0.4$  ml/min/g kidney wt after equilibration,  $9.4 \pm 0.4$  ml/min/g kidney wt at 120 min).

Experimental groups were as follows. All experiments in series 1 and 2 were carried out two weeks after the injection of STZ.

#### Series 1

The aim of this experiment was to evaluate the acute effects of specific TXA<sub>2</sub> inhibition on the renal hemodynamics in normal and diabetic rats. In order to inhibit the activity of TXA<sub>2</sub>, a specific TXA<sub>2</sub> synthetase inhibitor, (E)-3-[4-(1-imidazolylmethyl)phenyl]-2-propenoic acid hydrochloride monohydrate (OKY-046, Ono Pharmaceuticals, Osaka, Japan) or a specific TXA<sub>2</sub> receptor antagonist, 4-[2-(benzene-sulfonamido)ethyl]-phenoxyacetic acid (BM13.177, Sulotroban, Yamanouchi Pharmaceuticals, Tokyo, Japan) was used. OKY-046 was dissolved into physiological saline and administered at the rate of 6 mg/kg/hr (1 ml/hr). Sulotroban was dissolved into 4.04% l-arginine solution and administered at the rate of 20 mg/kg/hr (1 ml/hr). Four groups of rats were studied: N-OKY ( $N = 6$ ), normal control rats treated with OKY-046; DM-OKY ( $N = 10$ ), diabetic rats treated with OKY-046; DM-Sult ( $N = 8$ ), diabetic rats treated with Sulotroban; DM-C ( $N = 8$ ), diabetic rats treated with physiological saline ( $N = 4$ ) or 4.04% l-arginine solution ( $N = 4$ ). Because neither physiological saline nor 4.04% l-arginine solution had any effect on renal hemodynamics, the data were assembled into one group (DM-C). Sixty minutes after the flow probe was placed on the left renal artery, RBF was measured every ten minutes during a basal period of

30 minutes (control period) and during a subsequent experimental period of 30 minutes (experimental period) that commenced 30 minutes after starting an intravenous infusion of OKY-046, Sulotroban or vehicles. Urine samples were collected into plastic tubes during each period and frozen at  $-20^{\circ}\text{C}$  until the measurements of PGE<sub>2</sub>, 6-keto-PGF<sub>1 $\alpha$</sub>  and TXB<sub>2</sub>. About 0.1 ml of blood samples were collected from the left femoral artery at the beginning and the end of each period, that is, the total blood volume drawn was 0.4 ml in each rat, to determine hematocrits and fasting blood glucose concentrations, and were replaced with the same volume of physiological saline.

#### Series 2

The aim of this experiment was to evaluate the influence of an angiotensin converting enzyme (ACE) inhibitor or an angiotensin II receptor antagonist on the acute effect of OKY-046 on renal hemodynamics. Two groups of diabetic rats were studied to evaluate the influence of an ACE inhibitor: DM-MK ( $N = 9$ ), diabetic rats treated with the ACE inhibitor, MK422 (enalaprilat, Banyu Pharmaceuticals, Tokyo, Japan); and DM-MK-OKY ( $N = 10$ ), diabetic rats treated with OKY-046 after the administration of MK422. In DM-MK, MK422 was dissolved into physiological saline and administered at the rate of 1 mg/kg/hr (1 ml/hr). Measurements of RBF and collections of urine and blood samples were made on the same protocol as in series 1. In DM-MK-OKY, the experiment was also performed on the same protocol as in series 1, but the infusion of MK422 at the same rate in DM-MK was initiated 30 minutes before the measurement of the control period and continued throughout the experiment. All values obtained in DM-MK-OKY group, therefore, were under the condition of ACE inhibition. Two other groups of rats were studied to evaluate the influence of an angiotensin II receptor antagonist on the effect of OKY-046 on renal hemodynamics. An angiotensin II receptor antagonist, Saralasin (Sigma, St. Louis, Missouri, USA), was used. Six diabetic rats (DM-Sara-OKY) and four normal control rats (N-Sara-OKY) were studied on the same protocol as in series 1. After measurements in the control period, Saralasin at the rate of 0.3 mg/kg/hr and OKY-046 at the same rate in DM-OKY were initiated simultaneously and continued throughout the experiment. Measurements of RBF in the experimental period were performed after 30 minutes of equilibration. This dose of Saralasin blocked the pressor response to an acute intravenous bolus injection of angiotensin II sufficient to raise the mean arterial pressure (MAP) by at least 25 mm Hg [17].

#### Series 3

To evaluate the acute effects of specific TXA<sub>2</sub> inhibition on the renal hemodynamics in long-term untreated diabetic rats, renal hemodynamic study was performed in 28-week-old diabetic rats ( $N = 5$ ) before and after the administration of OKY-046 on the same protocol as in DM-OKY group. They received no insulin and were allowed free access to water and a regular rat pellet diet for 28 weeks.

#### Series 4

The effects of chronic TXA<sub>2</sub> inhibition on the progression of diabetic renal abnormalities were evaluated. Three groups of rats were studied: DM-T ( $N = 12$ ), diabetic rats treated with OKY-046 in the drinking water (80 mg/kg/day) for six months;

**Table 1.** Body weight (body wt), left kidney weight (kidney wt), fasting blood glucose concentration (FBS), hematocrit (Hct), and mean arterial pressure (MAP) in each group in series 1 and 2

	N	Body wt	Kidney wt	FBS mg/dl	Hct %		MAP mm Hg		
		g			cont.	exper.	cont.	exper.	
Series 1									
N-OKY	5	239 ± 4 <sup>b</sup>	1.1 ± 0.03	98 ± 7	43.8 ± 0.7	43.4 ± 1.3	110 ± 4	111 ± 4	
DM-C	8	243 ± 11 <sup>b</sup>	1.4 ± 0.1 <sup>a</sup>	333 ± 10 <sup>a,b</sup>	45.6 ± 1.4 <sup>a</sup>	45.5 ± 1.5	112 ± 4 <sup>b</sup>	114 ± 3	
DM-OKY	10	251 ± 11 <sup>b</sup>	1.4 ± 0.05 <sup>a</sup>	262 ± 22 <sup>a,b</sup>	47.2 ± 0.7 <sup>a</sup>	46.5 ± 0.6	109 ± 3 <sup>b,e</sup>	110 ± 2	
DM-Sult	8	247 ± 9 <sup>b</sup>	1.5 ± 0.1 <sup>a</sup>	291 ± 19 <sup>a,b</sup>	50.2 ± 0.7 <sup>a,c,d</sup>	49.8 ± 0.8	108 ± 2 <sup>b</sup>	111 ± 2	
Series 2									
DM-MK	9	270 ± 8	1.5 ± 0.1 <sup>a,b</sup>	264 ± 19 <sup>a,b</sup>	49.2 ± 0.8 <sup>a,c,d</sup>	49.1 ± 0.8	118 ± 2	116 ± 1 <sup>f</sup>	
DM-MK-OKY	10	260 ± 12 <sup>b</sup>	1.5 ± 0.1 <sup>a,b</sup>	250 ± 19 <sup>a,b</sup>	50.6 ± 0.5 <sup>a,c,d</sup>	50.1 ± 0.6	110 ± 2 <sup>b,e</sup>	112 ± 2	
DM-Sara-OKY	6	269 ± 10	1.5 ± 0.07 <sup>a,b</sup>	232 ± 27 <sup>a,b</sup>	50.6 ± 0.5 <sup>a,c,d</sup>	50.1 ± 0.6	110 ± 2 <sup>b,e</sup>	112 ± 2	
N-Sara-OKY	4	296 ± 25	1.2 ± 0.1	98 ± 6	49.1 ± 0.7 <sup>a,c,d</sup>	47.7 ± 0.5	124 ± 6	119 ± 4	

Values are expressed as means ± SEM. Abbreviations are: N, number of rats; cont., control period; exper., experimental period.

Treatment regimen abbreviations are: N-OKY, age-matched normal rats treated with thromboxane synthetase inhibitor (TSI); DM-C, 2-week-old diabetic rats treated with vehicles; DM-OKY, 2-week-old diabetic rats treated with TSI; DM-Sult, 2-week-old diabetic rats treated with TXA<sub>2</sub> receptor antagonist (TR); DM-MK, 2-week-old diabetic rats treated with ACE inhibitor; DM-MK-OKY, 2-week-old diabetic rats treated with TSI and ACE inhibitor; DM-Sara-OKY, 2-week-old diabetic rats treated with TSI and angiotensin II receptor antagonist (AR); N-Sara-OKY, age-matched normal rats treated with TSI and AR.

<sup>a</sup> *P* < 0.05 vs. N-OKY

<sup>b</sup> *P* < 0.05 vs. N-Sara-OKY

<sup>c</sup> *P* < 0.05 vs. DM-C

<sup>d</sup> *P* < 0.05 vs. DM-OKY

<sup>e</sup> *P* < 0.05 vs. DM-MK

<sup>f</sup> *P* < 0.05 vs. respective control period

DM (*N* = 10), diabetic rats treated with vehicle alone; Normal (*N* = 8), age-matched normal rats treated with vehicle alone. Diabetic rats received no insulin. OKY-046 was dissolved into distilled water and prepared every two days. Water intake was measured every two weeks and the concentration of OKY-046 was adjusted. OKY-046 was initiated three days after the induction of diabetes. Fed blood glucose concentration and body weight were also measured every two weeks. Twenty-four-hour urine collections were performed at 2, 4, 8, 12, and 24 weeks after the initiation of OKY-046 treatment to measure urinary albumin excretion (UAE), urinary TXB<sub>2</sub> excretion, and urinary 6-keto-PGF<sub>1α</sub> excretion. Measurements of RBF and inulin clearance (GFR) were performed 2 and 24 weeks after the initiation of OKY-046 treatment. Surgical procedures and fluid replacements were the same as those in series 1, but physiological saline and 2% inulin solution, dissolved into physiological saline, were infused at a rate of 1 ml/hr during the experiment. Sixty minutes after surgery, three 15-minute timed urine collections were performed and 0.1 ml of blood samples were collected at the midpoint of urine collection. RBF was measured every 15 minutes.

#### Analytical procedures

Renal vascular resistance (RVR) and filtration fraction (FF) were calculated as follows: RVR = MAP/RBF, FF = GFR/RBF(1-Hct). Blood glucose concentration was determined with glucose oxydase method. Inulin concentration was measured with photometry by use of a cysteine-tryptophan reaction [45] in order to avoid the influence of the chromogens, such as glucose. Coefficient of variation at our laboratory was less than 5%. Urinary albumin concentration was determined by enzyme-linked immunosorbent assay (Ono Pharmaceuticals). Urinary PGE<sub>2</sub>, 6-keto-PGF<sub>1α</sub> and TXB<sub>2</sub> were extracted as follows [11]. The urine samples were acidified to pH 3.0 with 1 N HCl,

and ethanol was added to 15% (vol/vol). The aliquots were applied onto the ODS-silica columns (SEP-PAK C18 cartridge, Waters Associates, Milford, Massachusetts, USA) and eluted with 10 ml of 15% ethanol followed by 10 ml of distilled water, 20% chloroform and n-hexane. PGs and TXs absorbed to the column were eluted with 10 ml of methylformate, collected, and dried under a stream of nitrogen gas. This fraction was reconstituted in 1 ml of phosphate-buffered saline solution (pH 7.3) and then PGE<sub>2</sub>, 6-keto-PGF<sub>1α</sub> and TXB<sub>2</sub> in the reconstituted fraction were measured by radioimmunoassay with specific antisera (anti-PGE<sub>2</sub>: Amersham Japan, Tokyo, Japan; anti-6-keto-PGF<sub>1α</sub> and anti-TXB<sub>2</sub>: Ono Pharmaceuticals). The percentage of recovery calculated by using radioactive PGE<sub>2</sub>, 6-keto-PGF<sub>1α</sub> and TXB<sub>2</sub> (Ono Pharmaceuticals) was almost 90%.

#### Statistical analysis

In every rat all values obtained during each period were averaged, and the paired Student's *t*-test was carried out comparing the mean values of the control periods to subsequent experimental periods. Differences among groups were determined by one-way analysis of variance (one-way ANOVA). All values are reported as means ± SEM. *P* values of <0.05 were considered to be statistically significant.

#### Results

Body weight, left kidney weight, fasting blood glucose concentration (FBS), hematocrit (Hct) and mean arterial pressure (MAP) in each group in series 1 and 2 are shown in Table 1. All diabetic rats gained body weight. Body weights were not different among the diabetic groups. The kidney weight in each diabetic group was significantly greater than that in normal rats. Non-fasting blood glucose concentration on the day before the experiment was 441 ± 83 mg/dl in all of the



**Table 2.** Changes in renal blood flow (RBF) and renal vascular resistance (RVR) in each group in series 1 and 2

	RBF ml/min		RVR mm Hg/ml/min	
	cont.	exper.	cont.	exper.
<b>Series 1</b>				
N-OKY	8.4 ± 0.4	8.9 ± 0.5	13.2 ± 0.8	12.5 ± 1.0
DM-C	11.0 ± 0.8 <sup>a</sup>	11.1 ± 0.8	10.7 ± 0.8 <sup>a</sup>	10.6 ± 0.8
DM-OKY	11.8 ± 0.6 <sup>a</sup>	10.3 ± 0.6 <sup>c</sup>	9.5 ± 0.6 <sup>a</sup>	11.0 ± 0.8 <sup>c</sup>
DM-Sult	11.7 ± 0.3 <sup>a</sup>	11.1 ± 0.4 <sup>c</sup>	9.3 ± 0.3 <sup>a</sup>	9.9 ± 0.4 <sup>c</sup>
<b>Series 2</b>				
DM-MK	11.8 ± 0.5 <sup>a</sup>	12.2 ± 0.5	10.2 ± 0.5 <sup>a</sup>	9.9 ± 0.5
DM-MK-OKY	13.3 ± 0.7 <sup>a,b</sup>	12.9 ± 0.7	8.5 ± 0.5 <sup>a</sup>	8.7 ± 0.5
DM-Sara-OKY	11.9 ± 0.8 <sup>a</sup>	11.8 ± 0.7	9.7 ± 0.5 <sup>a</sup>	9.7 ± 0.5
N-Sara-OKY	10.6 ± 0.7	10.6 ± 0.7	11.9 ± 1.0	11.5 ± 1.0

Values are expressed as means ± SEM.

<sup>a</sup> *P* < 0.05 vs. N-OKY

<sup>b</sup> *P* < 0.05 vs. N-Sara-OKY

<sup>c</sup> *P* < 0.05 vs. respective control period

Abbreviations and treatment regimen abbreviations are in Table 1.

diabetic rats. Ten to 14 hours fasting reduced blood glucose concentrations, but hyperglycemia was still observed in each diabetic group. None of the diabetic rats had ketonuria after 10 to 14 hours of fasting. Initial Hcts in DM-Sult, DM-MK, DM-MK-OKY, DM-Sara-OKY and N-Sara-OKY were higher than those in N-OKY, DM-C and DM-OKY, but the Hct in each group were stable throughout the experiment. Initial MAP in N-Sara-OKY was higher than those in other groups except DM-MK and DM-Sara-OKY. MAP in all groups except DM-MK was unchanged throughout the experiment. In DM-MK, MAP was slightly decreased after the administration of MK422.

RBF and RVR in series 1 and 2 are shown in Table 2. Diabetic rats had significantly higher RBF and lower RVR than normal rats in the control period. RBF and RVR in the control period were not different among diabetic groups.

#### Series 1

The acute effects of specific TXA<sub>2</sub> inhibition on the renal hemodynamics were studied in normal and two-week-old diabetic rats. RBF and RVR were not changed after the administration of a specific TXA<sub>2</sub> synthetase inhibitor, OKY-046, in normal control rats (N-OKY). In diabetic rats, the administration of OKY-046 significantly decreased RBF and increased RVR (DM-OKY). A specific TXA<sub>2</sub> receptor antagonist, Sulotroban, also decreased RBF and increased RVR slightly but significantly (DM-Sult). Both OKY-046 and Sulotroban, however, did not completely restore RBF and RVR to the normal control values. OKY-046 decreased RBF from 140% to 116% of normal control value, and Sulotroban decreased RBF from 139% to 124%. The administration of vehicle alone did not cause any change in RBF and RVR in normal control rats (Methods) and diabetic rats (DM-C).

#### Series 2

The influence of ACE inhibition or angiotensin II receptor blockade on the acute effect of OKY-046 on the renal hemodynamics was examined in two-week-old diabetic rats. The administration of ACE inhibitor, MK422, alone did not cause any change in RBF and RVR. In DM-MK-OKY, in which the

infusion of OKY-046 was initiated 60 minutes after starting the continuous infusion of MK422, the administration of OKY-046 did not cause any decrease of RBF or any increase of RVR, as was observed in DM-OKY. In addition, the simultaneous infusion of Saralasin with OKY-046 (DM-Sara-OKY) blunted the renal vasoconstrictive effect of OKY-046 observed in DM-OKY. Simultaneous infusion of Saralasin with OKY-046 did not affect the renal hemodynamics in normal control rats (N-Sara-OKY).

The changes in RBF (d-RBF) and in RVR (d-RVR) after the administration of vehicles, OKY-046, Sulotroban, MK422 +OKY-046 or Saralasin+OKY-046 in two-week-old diabetic rats are shown in Figures 1 and 2. d-RBF and d-RVR after the administration of OKY-046 or Sulotroban were significantly larger than those after the administration of vehicles. d-RBF and d-RVR after the administration of MK422+OKY-046 or Saralasin+OKY-046 were significantly smaller than those after the administration of OKY-046, and were not different from those after the administration of vehicles.

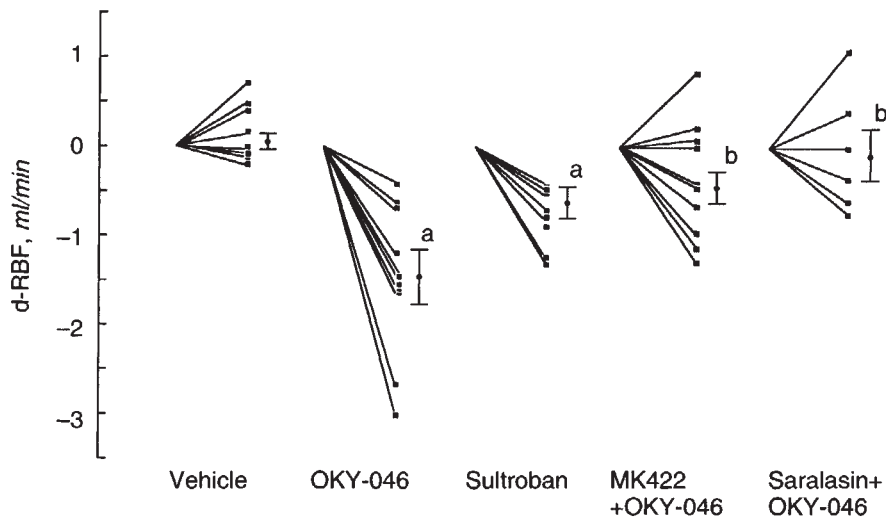
Urinary excretion rates of TXB<sub>2</sub>, PGE<sub>2</sub> and 6-keto-PGF<sub>1α</sub> in DM-OKY, DM-Sult, DM-MK and DM-MK-OKY are shown in Table 3. In DM-OKY, the urinary excretion rate of TXB<sub>2</sub> was significantly decreased after the administration of OKY-046, but those of PGE<sub>2</sub> and 6-keto-PGF<sub>1α</sub> were not changed. Sulotroban and MK422 did not affect the urinary excretion rates of TXB<sub>2</sub> and PGE<sub>2</sub>. In DM-MK-OKY, the urinary excretion rate of TXB<sub>2</sub> was significantly decreased but that of PGE<sub>2</sub> was not changed.

#### Series 3

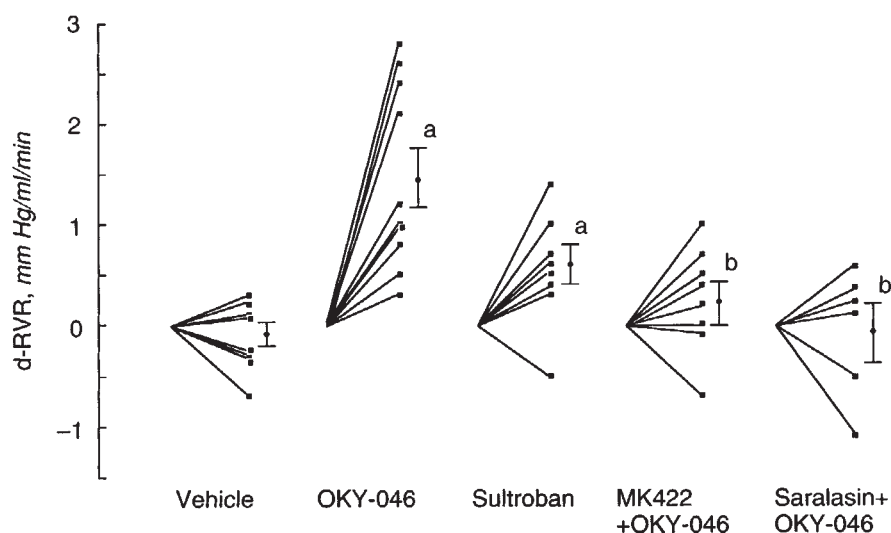
The acute effects of specific TXA<sub>2</sub> inhibition on the renal hemodynamics in 28-week-old diabetic rats were studied. The fed blood glucose concentrations were more than 400 mg/dl and they had no ketonuria. Body weight was gradually increased but significantly lower than that in age-matched normal control rats at 28 weeks (diabetic, 316 ± 12; normal, 548 ± 11 g; *P* < 0.01). Left kidney weight in diabetic rats was larger than that in normal control rats (diabetic, 2.3 ± 0.1; normal, 1.8 ± 0.1 g; *P* < 0.01). Table 4 shows Hct, MAP, RBF, and RVR before and after the administration of OKY-046. Hct and MAP were unchanged during the renal hemodynamic study. The administration of OKY-046 significantly increased RBF and decreased RVR, as opposed to the results observed in two-week-old diabetic rats (Table 2, DM-OKY), but OKY-046 did not completely restore RBF and RVR.

#### Series 4

The effects of long-term TXA<sub>2</sub> inhibition on the progression of renal abnormalities in diabetic rats were examined. The data were summarized in Table 5. In the diabetic groups, fed blood glucose concentrations were more than 400 mg/dl and ketonuria was not detected during six months. They gradually gained body weight. The body weight and left kidney weight in the diabetic groups were not different between the groups but significantly different from those in normal rats. MAP at 2 weeks and 24 weeks was not different among three groups. Urinary excretions of TXB<sub>2</sub> and PGF<sub>1α</sub> in non-treated diabetic rats (DM group) were significantly increased both at 2 and 24 weeks. Chronic administration of OKY-046 in DM-T group specifically decreased urinary TXB<sub>2</sub> excretion.



**Fig 1.** Changes in renal blood flow (d-RBF) after infusion of vehicle, OKY-046, Sultroban, MK422+OKY-046, and Saralasin+OKY-046 in 2-week-old diabetic rats. d-RBF after infusion of OKY-046 or Sultroban was larger than that after infusion of vehicles. d-RBF after infusion of MK422+OKY-046 or Saralasin+OKY-046 was significantly smaller than that after infusion of OKY-046 but not different from that after infusion of vehicle. (a)  $P < 0.05$  vs. vehicle; (b)  $P < 0.05$  vs. OKY.



**Fig 2.** Changes in renal vascular resistance (d-RVR) after infusion of vehicle, OKY-046, Sultroban, MK422+OKY-046, and Saralasin+OKY-046 in 2-week-old diabetic rats. d-RVR after infusion of OKY-046 or Sultroban was larger than that after infusion of vehicles. d-RVR after infusion of MK422+OKY-046, or Saralasin+OKY-046 was significantly smaller than that after infusion of OKY-046 but not different from that after infusion of vehicle. (a)  $P < 0.05$  vs. vehicle; (b)  $P < 0.05$  vs. OKY.

**Table 3.** Changes in urinary excretion of TXB<sub>2</sub>, PGE<sub>2</sub>, 6-keto-PGF<sub>1α</sub> in each group in series 1 and 2

	N	TXB <sub>2</sub> pg/min		PGE <sub>2</sub> pg/min		6-keto-PGF <sub>1α</sub> pg/min	
		cont.	exper.	cont.	exper.	cont.	exper.
DM-OKY	5	58.5 ± 5.6	33.6 ± 7.2 <sup>a</sup>	44.6 ± 5.4	31.5 ± 7.6	62 ± 13.0	136 ± 23.8
DM-Sult	5	63.6 ± 12.3	45.9 ± 8.5	43.7 ± 43.6	43.6 ± 8.8	ND	ND
DM-MK	4	49.6 ± 8.6	37.0 ± 4.5	70.6 ± 10.4	51.5 ± 16.4	102 ± 16.9	79 ± 4.5
DM-MK-OKY	3	39.8 ± 5.8	18.4 ± 3.2 <sup>a</sup>	97.9 ± 20.9	71.4 ± 11.0	ND	ND

Values are expressed as means ± SEM. ND is not done. See Table 1 for abbreviations.

<sup>a</sup>  $P < 0.01$  vs. respective control period

At two weeks, DM group showed hyperperfusion, hyperfiltration, and increased UAE, compared with normal rats. RBF and RVR in DM-T were completely restored to those in normal rats. Although GFR in DM-T was slightly lower and FF was slightly higher than those in DM group, these differences were not statistically significant. UAE in DM-T was significantly lower than that in DM group but still higher than that in normal rats.

At 24 weeks, the DM group showed hypoperfusion and normal glomerular filtration rate, so that FF was significantly higher than that in normal rats. Chronic administration of OKY-046 in DM-T group ameliorated the hypoperfusion by decreasing RVR. GFR in DM-T was slightly lower than that in DM group. FF in DM-T group was completely restored to the normal control value. UAE in DM-T group was remarkably lower than that in DM group.

**Table 4.** Effects of acute administration of TXA<sub>2</sub> synthetase inhibitor, OKY-046, on renal hemodynamics in 28-week-old diabetic rats

	Hct	MAP	RBF	RVR
Control period	50.5 ± 1.1	112 ± 4	9.3 ± 0.8	12.5 ± 1.3
Experimental period	50.0 ± 1.3	114 ± 2	10.2 ± 0.7 <sup>a</sup>	11.5 ± 1.0 <sup>a</sup>

Values are expressed as means ± SEM. See Tables 1 and 2 for abbreviations.

<sup>a</sup>  $P < 0.05$  vs. control period

### Discussion

In this study, untreated diabetic rats at two weeks after the induction of diabetes showed renal hyperperfusion, hyperfiltration and normal FF, whereas the diabetic rats at 24 weeks showed hypoperfusion, normal GFR and increased FF. These data were consistent with previous findings [18–21]. Since MAP is stable, it is suggested that RVR is a major factor in the changes of RBF.

The new findings of this study were that an administration of specific TXA<sub>2</sub> synthetase inhibitor, OKY-046, or specific TXA<sub>2</sub> receptor antagonist, Sulotroban, increased RVR and ameliorated the hyperperfusion at the early stage (2 weeks) of untreated diabetic rats, whereas OKY-046 decreased RVR and ameliorated the renal hypoperfusion at the late stage (24 to 28 weeks) of untreated diabetic rats. In addition, it is also new that the renal vasoconstrictive effect of OKY-046 at the early stage of untreated diabetic rats was blunted under the condition of renin-angiotensin blockade. Three lines of evidence suggest that the changes in renal hemodynamics after administration of OKY-046 or Sulotroban in early untreated diabetic rats are most likely related to the specific inhibition of TXA<sub>2</sub> activity. First, RBF and RVR in DM-C, which served as the time-controls, were unchanged throughout the experiment. Second, the observation that urinary excretions of PGE<sub>2</sub> and 6-keto-PGF<sub>1α</sub> were unchanged in spite of the significant decreases of urinary excretion of TXB<sub>2</sub> in DM-OKY and DM-MK-OKY indicated that OKY-046 selectively inhibited TXA<sub>2</sub> synthetase. Third, the TXA<sub>2</sub>/PGE<sub>2</sub> receptor antagonist, Sulotroban, also increased RVR and decreased RBF in diabetic rats, indicating that PGE<sub>2</sub> was not involved in the renal vasoconstriction. To our knowledge, Sulotroban has no agonistic actions at the dose used in this study. Our preliminary study demonstrated that the dose of Sulotroban used in this study was enough to inhibit the vasoconstriction caused by TXA<sub>2</sub> mimetic, U46619 (data not shown). It was not likely that the renal vasoconstrictive effect was secondary to the changes of MAP, since neither OKY-046 nor Sulotroban affected MAP. It is therefore suggested that the specific TXA<sub>2</sub> inhibition causes renal vasoconstriction at the early stage, but causes renal vasodilation at the late stage of untreated diabetic rats.

The renal vasoconstrictive effect of OKY-046 in early untreated diabetic rats blunted under the condition of ACE inhibition. ACE inhibitors may potentiate the activity of bradykinin and increase the production of vasodilatory PGs. MK422 did not affect the urinary excretions of 6-keto-PGF<sub>1α</sub> and PGE<sub>2</sub> in this study. It has been reported that MK422 may have some effects on kininase activity, but blockade of the renin-angiotensin system accounts for a significant part of the effects of

MK422 [22]. In addition, the fact that Saralasin also blunted the renal vasoconstrictive effects of OKY-046 at the early stage of diabetic rats indicated that angiotensin II was involved in the mechanisms of renal vasoconstriction. These findings indicate that OKY-046 may affect the renal hemodynamics at the early stage of diabetic rats through mediation of the renin-angiotensin system. It is suggested that increased TXA<sub>2</sub> at the early stage of untreated diabetic rats might contribute to the hyperperfusion through the mechanism in which increased TXA<sub>2</sub> causes the relative suppression of angiotensin II activity. However, other factors, such as vasodilatory PGs [3–9] and atrial natriuretic peptide [23, 24], also contribute to the hyperperfusion, because the complete reversal of diabetic hyperperfusion was not achieved by TXA<sub>2</sub> synthetase inhibition or TXA<sub>2</sub> receptor blockade in this study.

The relationships between TXA<sub>2</sub> and renin-angiotensin system have been reported by some investigators [25–27]. In diabetic patients, the furosemide stimulated increase of plasma renin activity (PRA) is less than that in normal subjects, and there is a significant negative correlation between PRA and urinary TXB<sub>2</sub>/6-keto-PGF<sub>1α</sub> ratio, suggesting that relatively increased TXA<sub>2</sub> could be a factor in the lower response in PRA [25]. Welch, Wilcox and Dunbar [26] clearly demonstrated that PRA was increased by the administration of TXA<sub>2</sub> synthetase inhibitor or TXA<sub>2</sub> receptor antagonist and was decreased by TXA<sub>2</sub> mimetic in anesthetized normal rats, and that a rise in PRA with TXA<sub>2</sub> antagonist was not clearly related to changes in salt balance, blood pressure or GFR. Omoto et al [27] reported that intraperitoneal administration of OKY-046 augmented renal cortical renin release and increased PRA in untreated STZ-induced diabetic rats. Our preliminary study revealed that the diabetic rats which were treated with oral administration of OKY-046 (80 mg/kg/day) for two weeks had lower RBF and higher PRA than untreated diabetic rats (RBF: 8.3 ± 0.3 vs. 11.7 ± 0.6 ml/min,  $P < 0.01$ ; PRA: 4.5 ± 0.2 vs. 3.6 ± 0.4 ng/ml/hr; 0.1 <  $P < 0.05$ , respectively,  $N = 5$  in each group). It is therefore suggested that the inhibition of TXA<sub>2</sub> activity at the early stage of diabetic rats must cause the enhancement of angiotensin II activity, of which the vasoconstrictive effect on renal vascular beds could surpass the vasodilatory effect caused by the TXA<sub>2</sub> inhibition itself, resulting in the amelioration of hyperperfusion.

The role of TXA<sub>2</sub> on renal hemodynamics in normal and pathologic conditions has been investigated [28, 29]. TXA<sub>2</sub> appears to be of little importance in the maintenance of renal function under physiological conditions, since the inhibition of TXA<sub>2</sub> does not alter GFR or RBF [28, 29]. Our results that OKY-046 did not affect renal hemodynamics in normal control rats confirmed the previous reports [28, 29]. However, Welch and Wilcox reported that the TXA<sub>2</sub> synthetase inhibitor, CGS-13080, but not the TXA<sub>2</sub> receptor antagonist, SQ-29548, reduced the clearance of p-aminohippurate (PAH) in normal control rats [30]. Tucker et al demonstrated that TXA<sub>2</sub> synthetase inhibitor, Dazmegrel, decreased single nephron plasma flow and nephron filtration rate in normal control rats. They suggested that other vasoactive agents might participate during TXA<sub>2</sub> synthetase inhibitor infusion [31]. We do not have any explanation of this discrepancy in normal rat.

Increased renal TXA<sub>2</sub> production has been documented in various renal diseases, such as nephrotoxic serum nephritis,



**Table 5.** Effects of chronic administration of TXA<sub>2</sub> synthetase inhibitor, OKY-046, on renal hemodynamics and urinary albumin excretion in diabetic rats

	N	Body wt	Kidney wt	MAP	RBF	RVR
<b>2 weeks</b>						
Normal	3	303 ± 8	1.26 ± 0.03	104 ± 7	6.9 ± 0.6	18.4 ± 2.2
DM	5	244 ± 20 <sup>a</sup>	1.34 ± 0.02 <sup>a</sup>	113 ± 2	8.7 ± 0.5 <sup>a</sup>	13.1 ± 0.6 <sup>a</sup>
DM-T	6	243 ± 9 <sup>a</sup>	1.50 ± 0.09 <sup>a</sup>	115 ± 3	5.7 ± 0.4 <sup>c</sup>	20.8 ± 0.2 <sup>c</sup>
<b>24 weeks</b>						
Normal	5	548 ± 11	1.8 ± 0.01	120 ± 7	7.8 ± 0.8	15.5 ± 0.4
DM	5	316 ± 12 <sup>b,c</sup>	2.3 ± 0.10 <sup>a,e</sup>	112 ± 4	4.2 ± 0.4 <sup>b,e</sup>	27.9 ± 2.2 <sup>b,c</sup>
DM-T	6	319 ± 16 <sup>b,e</sup>	2.3 ± 0.10 <sup>a,e</sup>	112 ± 3	5.5 ± 0.1 <sup>a,c</sup>	20.7 ± 0.6 <sup>a,c</sup>

Values are expressed as means ± SEM.

<sup>a</sup> P < 0.05 vs. respective Normal

<sup>b</sup> P < 0.01 vs. respective Normal

<sup>c</sup> P < 0.05 vs. respective DM

<sup>d</sup> P < 0.01 vs. respective DM

<sup>e</sup> P < 0.01 vs. 2 week DM

Abbreviations are: N, numbers of rats; Body wt, body weight (g); Kidney wt, left kidney weight (g); RBF, renal blood flow (ml/min/g · kidney wt); RVR, renal vascular resistance (mm Hg/ml/min/g · kidney wt); GFR, glomerular filtration rate (ml/min/g · kidney wt); FF, filtration fraction; UAE, urinary albumin excretion (mg/day); U-TXB<sub>2</sub>, urinary TXB<sub>2</sub> excretion (ng/day); U-6keto-PGF<sub>1α</sub>, urinary 6-keto-PGF<sub>1α</sub> excretion (ng/day).

Treatment regimen abbreviations are: DM, diabetic rats treated with vehicle; DM-T, diabetic rats treated with TXA<sub>2</sub> synthetase inhibitor, OKY-046 (80 mg/kg/day).

**Table 5. Continued**

	GFR	FF	UAE	U-TXB <sub>2</sub>	U-6keto-PGF <sub>1α</sub>
<b>2 weeks</b>					
Normal	1.03 ± 0.13	0.30 ± 0.02	0.15 ± 0.03	6.1 ± 0.7	19.1 ± 3.3
DM	1.41 ± 0.27 <sup>a</sup>	0.33 ± 0.07	0.55 ± 0.08 <sup>a</sup>	14.2 ± 3.3 <sup>a</sup>	411.1 ± 64.9 <sup>b</sup>
DM-T	1.26 ± 0.11	0.44 ± 0.05	0.33 ± 0.04 <sup>a,c</sup>	6.4 ± 0.5 <sup>c</sup>	475.8 ± 35.2 <sup>b</sup>
<b>24 weeks</b>					
Normal	0.92 ± 0.4	0.20 ± 0.03	0.86 ± 0.49	7.6 ± 1.5	18.3 ± 2.4
DM	1.07 ± 0.12 <sup>c</sup>	0.46 ± 0.04 <sup>a</sup>	23.5 ± 6.5 <sup>b,e</sup>	33.3 ± 14.7 <sup>b,e</sup>	747.6 ± 130.5 <sup>b</sup>
DM-T	0.70 ± 0.1 <sup>c</sup>	0.22 ± 0.02 <sup>c</sup>	3.72 ± 1.04 <sup>b,d</sup>	4.1 ± 0.6 <sup>d</sup>	676.3 ± 80.9 <sup>b</sup>

cyclosporine nephropathy, renal allograft rejection and ureteral obstruction, and pharmacological inhibition of TXA<sub>2</sub> synthesis or TXA<sub>2</sub> receptor increase RBF and GFR [28, 29]. The effects of TXA<sub>2</sub> inhibition on renal hemodynamic abnormalities in diabetes have also been reported by some investigators [3, 6, 16, 32]. Renal plasma flow, but not GFR, increases after an administration of TXA<sub>2</sub> inhibitor in IDDM patients [16]. Craven et al [3, 6] reported that the administration of selective TXA<sub>2</sub> synthetase inhibitor enhanced hyperfiltration at four weeks and prevented the decline in GFR at eight weeks in untreated diabetic rats. An administration of TXA<sub>2</sub> receptor antagonist, SK&F96184, increases RBF, GFR and single nephron GFR in insulin-treated diabetic rats [32]. These findings suggest that increased TXA<sub>2</sub> in diabetes might serve as a simple vasoconstrictor designed to lower GFR and RBF. The results in this study that the acute and chronic TXA<sub>2</sub> inhibition caused renal vasodilatation at the late stage of diabetic rats are consistent with the previous findings. However, the results that the acute and chronic inhibition of TXA<sub>2</sub> caused renal vasoconstriction in two-week-old untreated diabetic rats contradict the previous reports [3, 6, 16, 32]. Tucker et al recently demonstrated that TXA<sub>2</sub> synthetase inhibitor, Dazmegrel, caused renal vasoconstriction in 7- to 10-day-old insulin-untreated diabetic rats [31] and in 70- to 100-day-old diabetic rats treated with small doses of insulin [33], which is consistent with our findings in 2-week-old diabetic rats. The discrepancy between our results in 2-week-old diabetic rats and previous reports [3, 6, 32] might

depend on the difference of the time when the experiments were performed. Renal hyperperfusion has been demonstrated at two weeks but not after four weeks in untreated diabetic rats [1, 2, 34–37], suggesting that the mechanisms responsible for renal hemodynamic abnormalities after four weeks might be different from those at two weeks. The responsiveness of vascular beds to angiotensin II is also different between early and late diabetic animals. The renal hemodynamic responses to exogenous or endogenous angiotensin II at early stages (1 to 2 weeks) of untreated diabetic rats are similar to [36, 38] or one half of those observed in normal rats [36, 39], while renal and aortic responsiveness to exogenous angiotensin II at late stage (4 to 12 weeks) of untreated diabetic rats are markedly diminished [39, 40]. Taking these observations into consideration, it is suggested that TXA<sub>2</sub> inhibition at the late stage could not result in an increase of RVR because of the diminished responsiveness to angiotensin II in renal vasculature, even if TXA<sub>2</sub> inhibition might enhance angiotensin II activity. TXA<sub>2</sub> inhibition at late stage, therefore, might simply cause the inhibition of the vasoconstrictive property of TXA<sub>2</sub> itself. The finding in this study that the acute administration of OKY-046 in 28-week-old untreated diabetic rats increased RBF and decreased RVR, reversed from the results in 2-week-old diabetic rats, supports the hypothesis. Insulin treatment might influence the effect of TXA<sub>2</sub> inhibition on diabetic renal hemodynamics. TXA<sub>2</sub> inhibition increases RBF in the early stage [32] but decreases RBF in the late stage [33] of insulin-treated diabetic rats. In contrast,

TXA<sub>2</sub> inhibition decreased RBF in the early stage but increased RBF in the late stage of insulin-untreated diabetic rats in this study. These paradoxical findings suggest that the duration of diabetes and insulin treatment are important considerations.

Chronic administration of OKY-046 significantly decreased UAE both at 2 weeks and 24 weeks, although it did not affect the kidney weight. Since OKY-046 ameliorated the hyperperfusion and slightly decreased GFR in 2-week-old diabetic rats, these renal hemodynamic changes might, at least partly, contribute to the decrease of UAE. Other factors, such as glomerular permeability, might also be involved in the decrease of UAE. In 24-week-old diabetic rats treated with OKY-046, the complete normalization of FF resulting from the improvement of the renal hypoperfusion by OKY-046 was observed. It has been reported that chronic administration of TXA<sub>2</sub> synthetase inhibitor prevented the glomerular basement membrane thickening [12], and the increases in glomerular volume and mesangial volume [41]. It is, therefore, suggested that both the ameliorations in the renal hemodynamic abnormalities and in the pathological changes caused by OKY-046 contribute to the decrease in UAE observed at the early and late stages of diabetic rats in this study.

In summary, the TXA<sub>2</sub> inhibition ameliorated not only the renal hyperperfusion at early stage but also the renal hypoperfusion at late stage of untreated diabetic rats. The effect of TXA<sub>2</sub> inhibition observed at the early stage of the diabetic rats was blunted by ACE inhibition or angiotensin II receptor blockade. It is suggested that TXA<sub>2</sub> might, at least in part, play an important role in the hyperperfusion by modulating the activity of the renin-angiotensin system at an early stage and also in the hypoperfusion at the late stage of untreated diabetic rats. In addition, the TXA<sub>2</sub> inhibition prevented the increase of UAE both at early and at late stages of untreated diabetic rats. These data support roles for TXA<sub>2</sub> in the progression of renal injury in STZ-induced diabetic rats. The specific mechanism by which the inhibition of TXA<sub>2</sub> enhances renin-angiotensin activity is not apparent in this study. Further studies are required to clarify this issue.

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