Effects of Pre- and Post-ischemic Treatments with FK409, a Nitric Oxide Donor, on Ischemia/Reperfusion-Induced Renal Injury and Endothelin-1 Production in Rats

Atsushi NAKAJIMA, Kyoko UEDA, Masanori TAKAOKA, Hayato KURATA, Junji TAKAYAMA, Mamoru OHKITA, and Yasuo MATSUMURA*

Department of Pharmacology, Osaka University of Pharmaceutical Sciences; 4–20–1 Nasahara, Takatsuki, Osaka 569–1094, Japan. Received November 16, 2005; accepted December 6, 2005; published online December 13, 2005

We have demonstrated that ischemic acute renal failure (ARF) is attenuated by pre-ischemic treatment with a spontaneous nitric oxide (NO) donor, (±)-(*E*)-4-ethyl-2-[(*E*)-hydroxyimino]-5-nitro-3-hexenamide (FK409). In the present study, we evaluated the effect of post-ischemic treatment with FK409 on ARF, compared with the pre-ischemic treatment effect. Ischemic ARF was induced by occlusion of the left renal artery and vein for 45 min followed by reperfusion, 2 weeks after contralateral nephrectomy. At 24 h after reperfusion, renal function in untreated ARF rats markedly decreased. In addition, increases in renal contents of endothelin-1 (ET-1), a deleterious mediator in the pathogenesis of ischemic ARF, were evident in untreated ARF rats at 24 h after reperfusion. Pre-ischemic treatment with FK409 (1 or 3 mg/kg, i.v.) at 5 min before ischemia attenuated ischemia/reperfusion-induced renal dysfunction and increased ET-1 contents after reperfusion. In contrast, post-ischemic treatment with FK409 (3 or 10 mg/kg, i.v.) at 6 h after reperfusion aggravated the renal injury, but did not affect the increased ET-1 content after reperfusion. These results suggest that pre-ischemic treatment with FK409 exerts renoprotective effects on ischemic ARF, probably through the suppression of renal ET-1 overproduction, whereas post-ischemic treatment with the NO donor worsens the ischemia/reperfusion-induced renal injury, through mechanisms unrelated to the ET-1 production after reperfusion.

Key words acute renal failure; ischemia; reperfusion; endothelin-1; nitric oxide

It has been reported that nitric oxide (NO) can reduce the endothelin-1 (ET-1) production in endothelial cells.[1,2] Mitsutomi *et al.*[2] examined various spontaneous NO donors and NO synthase (NOS) inhibitors on ET-1 production in porcine cultured endothelial cells, and found that NO donors suppressed ET-1 production, whereas NOS inhibitors increased ET-1 production. Thus, it is likely that NO has a crucial role in the regulation of ET-1 production and ET-1-related diseases. ET-1 participates in hypertension, vasospasm, atherosclerosis and ischemia/reperfusion injury.[3] In particular, there is growing evidence that ET-1 is involved in the development of ischemic acute renal failure (ARF), in which ET-1 content and ET-1 mRNA expression are elevated in post-ischemic kidneys.[4—6]

NO is synthesized in the kidney and plays an important role in regulating renal hemodynamics and function; however, the role of NO in ischemia/reperfusion-induced ARF is still controversial. It has been reported that a non-selective NOS inhibitor prevented hypoxia/reoxygenation injury in isolated rat proximal tubules, and sodium nitroprusside, a NO donor, or L-arginine, the NO precursor, enhanced the injury, thereby suggesting that NO is synthesized in proximal tubules and participates in tubular hypoxia/reoxygenation injury as one of the mediators.[7] In contrast, *in vivo* studies have shown a beneficial effect of NO in ischemic ARF models: the inhibition of NO production with a non-selective NOS inhibitor significantly deteriorated renal function of ischemic ARF rats, whereas the NOS inhibitor-induced renal dysfunction was abolished by L-arginine.[8] We have also found that renal dysfunction in ischemic ARF rats is markedly attenuated by pre-ischemic treatment with (±)-(*E*)-4-ethyl-2-[(*E*)-hydroxyimino]-5-nitro-3-hexenamide (FK409), a NO donor, thereby suggesting that pre-ischemic treatment with the NO donor has a protective effect against renal injury *in vivo* models of ischemic ARF[9]; however, it is unclear whether post-ischemic treatment with a NO donor could suppress the ischemia/reperfusion-induced renal dysfunction.

The purpose of the present study is to evaluate the effect of post-ischemic treatment with a NO donor on ischemia/reperfusion-induced renal dysfunction. Because ARF cannot be predicted in many clinical cases, it is more important to know whether the post-ischemic treatment is beneficial, at least enhances the recovery process, or is detrimental. Thus, we investigated the effects of post-ischemic treatment with FK409, a NO donor, and the findings were compared with those observed by the pre-ischemic treatment. In addition, we examined whether the effects of post-ischemic treatment with FK409 on the ischemic ARF would be associated with ischemia/reperfusion-induced renal overproduction of ET-1, a deleterious mediator in the pathogenesis of ischemic ARF.[10]

MATERIALS AND METHODS

Animal and Experimental Design Male Sprague–Dawley rats (280—300 g, 10 weeks of age, Japan SLC, Shizuoka, Japan) were used. The animals were housed in a light-controlled room with a 12-h light/dark cycle and were allowed *ad libitum* access to food and water. Experimental protocols and animal care methods in the experiments were approved by the Experimental Animal Committee at Osaka University of Pharmaceutical Sciences (Osaka, Japan). Two weeks before the study (at 8 weeks of age), the right kidney was removed through a small flank incision under pentobarbital anesthesia (50 mg/kg, i.p.). After a 2-week recovery period,

* To whom correspondence should be addressed. e-mail: matumrh@gly.oups.ac.jp

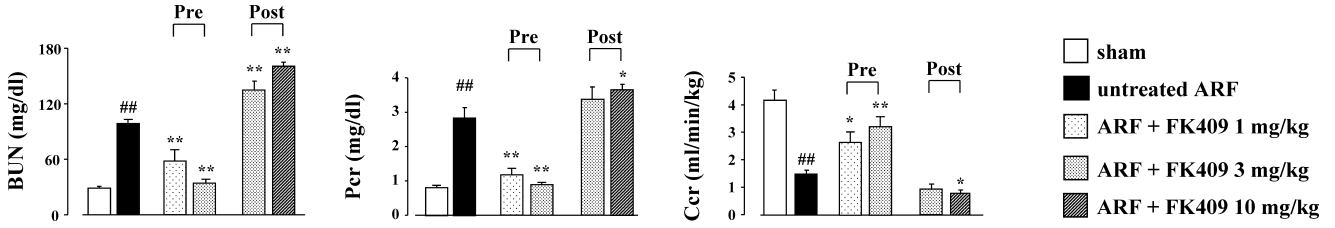

Fig. 1. Effect of Pre- or Post-ischemic Treatment with FK409 on Blood Urea Nitrogen (BUN), Plasma Creatinine Concentration (Pcr) and Creatinine Clearance (Ccr) at 24 h after Reperfusion

FK409 (1, 3 or 10 mg//kg) was given intravenously 5 min before ischemia (Pre) or 6 h after reperfusion (Post). Each column and bar represents the mean±S.E.M. (n=6). ARF, acute renal failure. ## $p<0.01$, compared with sham-operated rats. * $p<0.05$, ** $p<0.01$, compared with untreated ARF rats.

the rats were anesthetized with pentobarbital (50 mg/kg, i.p.), and the left kidney was exposed through a small flank incision. The left renal artery and vein were occluded with a nontraumatic clamp for 45 min. At the end of the ischemic period, the clamp was released to allow reperfusion. FK409 (1, 3 or 10 mg/kg) or vehicle (a mixture of 2.5% ethanol, 30% polyethylene glycol 400 and 67.5% saline) was administered (pre-ischemic treatment at 5 min before ischemia; post-ischemic treatment at 6 h after reperfusion) as a slow bolus injection at a volume of 1 ml/kg into the external jugular vein. In sham-operated control rats, the kidney was treated identically, except for the clamping. Animals exposed to 45-min ischemia were housed in metabolic cages 24 h after the ischemia. At the end of urine collection for 5 h, blood samples were drawn from the thoracic aorta, and then the left kidneys were excised under pentobarbital anesthesia (50 mg/kg, i.p.). The plasma was separated by centrifugation. These samples were used for measurement of renal function parameters.

Renal Function Parameters Blood urea nitrogen (BUN) and creatinine levels in plasma (Pcr) or urine were determined using a commercial assay kit, the BUN-test-Wako and Creatinine-test-Wako (Wako Pure Chemicals Industries, Osaka, Japan), respectively. Creatinine clearance (Ccr, ml/min/kg) was calculated from the formula Ccr=Ucr×UF/Pcr, where Ucr and Pcr are creatinine concentration in urine and plasma, and UF is urine flow.

Renal ET-1 Assay ET-1 was extracted from the kidney, according to the method described elsewhere.[11] Briefly, kidneys were weighed and homogenized for 1 min in 8 volumes of ice-cold organic solution (chloroform/methanol, 2 : 1, including 1 mM *N*-ethylmaleimide). The homogenates were left overnight at 4 °C and then 0.4 volumes of distilled water was added after which the homogenates were centrifuged at 1500×***g*** for 30 min and the supernatant was stored. Aliquots of the supernatant were diluted 1 : 10 with a 0.09% trifluoroacetic acid solution and applied to Sep-Pak C_{18} cartridges. The sample was eluted with 3 ml of 63.3% acetonitrile and 0.1% trifluoroacetic acid. Eluates were dried in a centrifugal concentrator, and the dried residue was reconstituted in an assay buffer for radioimmunoassay (RIA). The clear solution was subjected to RIA. Recoveries of ET-1 from renal tissue by this extraction procedure are approximately 80%. RIA for ET-1 was done, as described elsewhere,[11] using ET-1 antiserum (a generous gift from Dr. Marvin R. Brown, Department of Medicine, University of California, San Diego, CA, U.S.A.) that does not cross-react with big ET-1.

Drugs FK409, a kind gift from Fujisawa Pharmaceutical Co., Ltd. (Osaka, Japan), was dissolved in a mixture of 2.5% ethanol, 30% polyethylene glycol 400 and 67.5% saline just before administration. Other chemicals were obtained from Nacalai Tesque (Kyoto, Japan) and Wako.

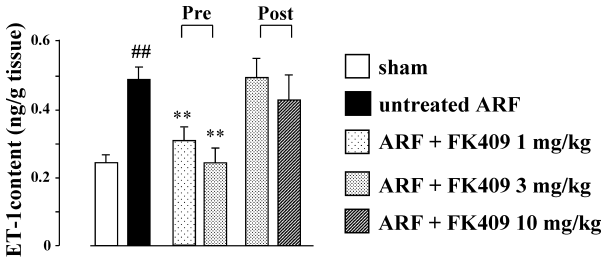

Fig. 2. Effect of Pre- or Post-ischemic Treatment with FK409 on Renal Endothelin-1 (ET-1) Contents at 24 h after Reperfusion

FK409 (1, 3 or 10 mg/kg) was given intravenously 5 min before ischemia (Pre) or 6 h after reperfusion (Post). Each column and bar represents the mean±S.E.M. (n=6). ARF, acute renal failure. ## $p<0.01$, compared with sham-operated rats. ** $p<0.01$, compared with untreated ARF rats.

Statistical Analysis Values were expressed as mean± S.E.M. The data were analyzed for significant differences between the sham-operated and untreated ARF groups using Student's unpaired t test. Statistical analysis for renal functional studies was performed using one-way analysis of variance followed by a Dunnett-type multiple comparison tests. For all comparisons, differences were considered significant at $p<0.05$.

RESULTS

Renal Function after the Ischemia/Reperfusion and Effect of Pre- or Post-ischemic Treatment with FK409 As shown in Fig. 1, renal function of rats subjected to a 45-min ischemia showed a marked deterioration when measured at 24 h after reperfusion. As compared with sham-operated rats, untreated ARF rats showed significant increases in BUN and Pcr, and a significant decrease in Ccr. Pre-ischemic treatment with FK409 (1, 3 mg/kg, i.v.) at 5 min before ischemia dose-dependently attenuated the ischemia/reperfusion-induced renal dysfunction. In contrast, post-ischemic treatment with FK409 (3, 10 mg/kg, i.v.) at 6 h after reperfusion aggravated the renal dysfunction. When FK409 was given at the higher dose, renal function changes induced by the ischemia/reperfusion were significantly worsened.

Renal ET-1 Content after the Ischemia/Reperfusion and Effect of Pre- or Post-ischemic Treatment with FK409 As shown in Fig. 2, renal ET-1 contents at 24 h after reperfusion were significantly increased in untreated ARF rats, compared

with those seen in sham-operated rats. The increases in ET-1 content were markedly suppressed by pre-ischemic treatment with FK409. On the other hand, post-ischemic treatment with FK409 unexpectedly did not affect the increased ET-1 contents, even at the higher dose.

DISCUSSION

The present study showed that pre-ischemic treatment with FK409 was capable of preventing the renal function impairment induced by ischemia/reperfusion. These results confirmed our previous findings that exogenous NO protects ischemia/reperfusion-induced renal injuries.[9] One can speculate that renal and/or systemic hemodynamic effects of FK409 given before the ischemia may influence the post-ischemic renal function. However, we observed that the pre-treatment with FK409 failed to ameliorate the decreased responses to renal plasma flow and glomerular filtration rate immediately after the reperfusion.[9] These findings suggest that the FK409-induced improvement of impaired renal function, observed at 24 h after the ischemia/reperfusion, is not due to acute renal hemodynamic changes, which might occur with FK409-induced renal vasodilation.

FK409 is reported to be a short-lived substance with the half-life of 46 min.[12] Our previous study found that the administration of FK409 (1, 3 mg/kg, i.v.) prior to ischemia resulted in a dose-related increase of NOx ($NO_2^- + NO_3^-$) in renal venous plasma.[9] These findings indicate that FK409 releases NO in the kidney during ischemia and immediately after reperfusion, and suggest that the FK409-induced improvement of impaired renal function, observed at 24 h after the ischemia/reperfusion, is due to NO released from FK409. Moreover, it seemed likely that NO released from FK409 improved ischemic ARF by preventing abnormal events occurring during a 45-min ischemia and an early phase after reperfusion; however, the mechanisms by which FK409 exhibits protective effects on ischemic ARF, were not elucidated in the previous study.

It has been demonstrated that ET-1 overproduction occurs at an early phase after the ischemia/reperfusion.[5,6] Wilhelm *et al.*[5] indicated that initial ET-1 up-regulation in the kidney occurs secondary to the ischemia, but reperfusion contributes to sustaining this up-regulation. In addition, they observed a marked increase of ET-1 in the peritubular capillary network, suggesting that ET-1-induced vasoconstriction plays a pathophysiological role in ischemia/reperfusion-induced tubular necrosis. Taken together, it is likely that increased local production of ET-1 and its action occur in the kidney after reperfusion. We also observed that renal ET-1 contents had already increased at 2 h after reperfusion.[6] Thus, the effectiveness of prior administration of FK409 to ischemic ARF rats is probably through the suppression of renal ET-1 overproduction in an early phase after reperfusion.

In the present study, we found that post-ischemic treatment with FK409 at 6 h after reperfusion aggravated the ischemia/reperfusion-induced renal dysfunction, in contrast to the administration of FK409 prior to the ischemia, and that the administration of FK409 after reperfusion could not augment the increased renal ET-1 content at 24 h after reperfusion, contrary to our expectations. These results indicate that FK409-derived NO exhibits opposite effects on ischemic ARF, dependently on the time of FK409 administration. In addition, it is conceivable that the post-ischemic treatment with FK409 does not affect the ET-1 production that once has been enhanced in an early phase after reperfusion.

We have recently obtained evidence that FK409 given at 6 h after reperfusion temporarily decreases the renal superoxide anion (O_2^-) level, and was followed by intense positive nitrotyrosine staining, suggesting the augmentation of peroxynitrite formation. (Nakajima *et al.*, unpublished data). Thus, it is likely that NO derived from FK409 given after reperfusion reacts with O_2^- to form peroxinitrite, which causes renal tissue damage *via* direct oxidation injury and protein tyrosine nitration.[13,14] Further experiments are required to clarify the deteriorating effect of FK409 on the ischemia/reperfusion-induced renal dysfunction when this agent was administered to ARF rats after reperfusion.

In conclusion, our results suggest that pre-ischemic treatment with FK409 exerts renoprotective effects on ischemic ARF, probably through the suppression of renal ET-1 overproduction, whereas post-ischemic treatment with FK409 worsens the ischemia/reperfusion-induced renal injury, through mechanisms unrelated to the ET-1 production after reperfusion.

Acknowledgment This work was supported by a "High-Tech Research Center" Project for Private Universities: matching fund subsidy from the ministry of Education, Culture, Sports, Science and Technology, Japan.

REFERENCES

1) Boulanger C., Lüscher T. F., *J. Clin. Invest.*, **85**, 587—590 (1991).
2) Mitsutomi N., Akashi C., Odagiri J., Matsumura Y., *Eur. J. Pharmacol.*, **364**, 65—73 (1999).
3) Schiffrin E. L., *Hypertension*, **25**, 1135—1143 (1995).
4) Firth J. D., Ratcliffe P. J., *J. Clin. Invest.*, **90**, 1023—1031 (1992).
5) Wilhelm S. M., Simonson M. S., Robinson A. V., Stowe N. T., Schulak J. A., *Kidney Int.*, **55**, 1011—1018 (1999).
6) Kuro T., Kohnou K., Kobayashi Y., Takaoka M., Opgenorth T. J., Wessale J. L., Matsumura Y., *Jpn. J. Pharmacol.*, **82**, 307—316 (2000).
7) Yu L., Gengaro P. E., Niederberger M., Burke T. J., Schrier R. W., *Proc. Natl. Acad. Sci. U.S.A.*, **91**, 1691—1695 (1994).
8) Chintala M. S., Chiu P. J., Vemulapalli S., Watkins R. W., Sybertz E. J., *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **348**, 305—310 (1993).
9) Matsumura Y., Nishiura M., Deguchi S., Hashimoto N., Ogawa T., Seo R., *J. Pharmacol. Exp. Ther.*, **287**, 1084—1091 (1998).
10) Takaoka M., Matsumura Y., *Recent. Res. Devel. Life Sci.*, **1**, 203—220 (2003).
11) Fujita K., Matsumura Y., Kita S., Miyazaki Y., Hisaki K., Takaoka M., Morimoto S., *Br. J. Pharmacol.*, **114**, 925—930 (1995).
12) Kato M., Nishino S., Ohno M., Fukuyama S., Kita Y., Hirasawa Y., Nakanishi I., Takasugi H., Sakane K., *Bioorg. Med. Chem. Lett.*, **6**, 33—38 (1996).
13) Beckman J. S., *Chem. Res. Toxicol.*, **9**, 836—844 (1996).
14) Radi R., Peluffo G., Alvarez M. N., Naviliat M., Cayota A., *Free Radic. Biol. Med.*, **30**, 463—488 (2001).