Preventive Effect of Lactacystin, a Selective Proteasome Inhibitor, on Ischemic Acute Renal Failure in Rats

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ABSTRACT
To elucidate the role of a proteasome-dependent proteolytic pathway in the pathogenesis of acute renal failure (ARF), we examined the effect of a selective proteasome inhibitor, lactacystin, on ARF induced by ischemia/reperfusion. Ischemic ARF was induced by clamping the left renal artery and vein for 45 min followed by reperfusion, 2 weeks after contralateral nephrectomy. Renal function in untreated ARF rats markedly decreased at 24 h after reperfusion. Intraperitoneal injection of lactacystin at a dose of 0.1 mg/kg before the occlusion tended to attenuate the deterioration of renal function. The higher dose of lactacystin (1 mg/kg) markedly attenuated the ischemia/reperfusion-induced renal dysfunction. Histopathological examination of the kidney of untreated ARF rats revealed severe lesions, such as tubular necrosis, proteinaceous casts in tubuli, and medullary congestion, all of which were markedly suppressed by the higher dose of lactacystin. In addition, endothelin (ET)-1 content in the kidney after the ischemia/reperfusion was significantly increased, being the maximum level at 6 h after the reperfusion, and this elevation was abolished by the higher dose of lactacystin. These results indicate that lactacystin prevents the development of ischemia/reperfusion-induced ARF, and the effect is accompanied by suppression of the enhanced ET-1 production in the kidney, thereby suggesting that a proteasome-dependent proteolytic pathway has a crucial role in the pathogenesis of ischemic ARF, possibly through the enhancement of ET-1 production in postischemic kidneys.

The proteasome is a multicatalytic proteinase complex present in cells as both 20S (700 kDa) and 26S (2000 kDa) forms. The 20S proteasome, with at least three distinct peptidase activities, i.e., trypsin-like, chymotrypsin-like, and peptidylglutamyl-peptide bond hydrolyzing activities, functions as the proteolytic core of the 26S proteasome complex that degrades ubiquitin-conjugated proteins (Coux et al., 1996; Tanaka, 1998). This ubiquitin-proteasome pathway is involved in the processing and degradation of regulatory proteins that control cell cycle progression (Glotzer et al., 1991), and in the activation process of a transcription factor, nuclear factor-κB (NF-κB) (Palombella et al., 1994; Traenckner et al., 1994); however, its pathophysiological role in vivo is still unclear.

Various substrate-related peptide aldehyde derivatives, such as acetyl-Leu-Leu-norleucinal, N-benzyloxy carbonyl (Cbz)-Leu-Leu-leucinal, Cbz-Leu-Leu-norvalinal (equivalent to calpain inhibitor-I, MG132, and MG115, respectively) and Cbz-Ile-Glu(O-t-Bu)-Ala-leucinal (called PSI), have been developed and used to clarify the physiological functions and pathophysiological roles of proteasome. They are known to inhibit not only proteasome but also cysteine proteinases (Coux et al., 1996; Tanaka, 1998). We recently found that PSI has preventive effects on ischemic acute renal failure (ARF) in rats (Takaoka et al., 1999). PSI is recognized as a potent and a cell-penetrating inhibitor of proteasome, but it does have weak calpain-inhibiting activity (Figueiredo-Pereira et al., 1994). Thus, one may point out that the preventive effect of PSI on ischemia/reperfusion-induced ARF is due to its inhibitory action on calpain.

In addition to synthetic peptide aldehyde inhibitors, there is a natural product inhibiting proteasome activity, which is known as lactacystin. Lactacystin, a microbial metabolite, was discovered by Omura et al. (1991) who isolated it from actinomycetes on the basis of its ability to induce neurite outgrowth in the murine neuroblastoma cell line. Subsequent work demonstrated that the biological effects of lactacystin result from its ability to inhibit proteasome, and this natural product is a potent and selective proteasome inhibitor that does not affect other proteinases examined so far (Fenteany et al., 1995). Thus, it seems reasonable to use lactacystin when evaluating pathophysiological roles of proteasome in many diseases, including ischemic ARF.

Previous studies have demonstrated that intracellular calcium, enzymes such as calpain, and several vasoactive sub-

ABBREVIATIONS: NF-κB, nuclear factor-κB; ARF, acute renal failure; ET, endothelin; Cbz, N-benzyloxy carbonyl; PSI, Cbz-Ile-Glu(O-t-Bu)-Ala-leucinal; BUN, blood urea nitrogen; Pcr, plasma creatinine concentration; Ccr, creatinine clearance; UF, urine flow; Uosm, urinary osmolality; FENa, fractional excretion of sodium; RIA, radioimmunoassay; TNF-α, tumor necrosis factor-α.
stances play an important role for the pathogenesis of ischemia/reperfusion injury of the kidney (Edelstein, 1997). Recently, there is growing evidence that endothelin (ET)-1 is involved in the development of ischemic ARF. The kidney is well known for synthesizing ET-1 and expressing both ET$_A$ and ET$_B$ receptors (Nambi et al., 1992). Both ET$_A$-selective and nonselective ET$_A$/ET$_B$ receptor antagonists have been reported to attenuate the ischemia/reperfusion-induced impairment of renal function (Mino et al., 1992; Gellai et al., 1995; Birck et al., 1998). It has furthermore been demonstrated that ET-1 content (Shibouta et al., 1990; Kuro et al., 2000) and ET-1 mRNA expression (Firth and Ratcliffe, 1992; Wilhelm et al., 1999) are elevated in postischemic kidneys. The mechanisms by which ET-1 production is enhanced in the kidney of ischemic ARF are obscure, whereas a recent report showing that PSI lessens the increased aortic ET-1 content in deoxycorticosterone acetate-salt hypertensive rats (Okamoto et al., 1998) intimates that there is a link between a proteasome-dependent proteolytic pathway and ET-1 production.

The purpose of the present study is to evaluate the effectiveness of selective proteasome inhibition on the ischemia/reperfusion-induced renal dysfunction and histological damage. To attain the objective, we decided to investigate the effect of lactacystin on the ischemia/reperfusion-induced renal injury and whether the effect would be accompanied by a decrease in renal ET-1 production.

Materials and Methods

Animals and Experimental Design. Male Sprague-Dawley rats (280–300 g, 10 weeks of age, Japan SLC, Inc., Hamamatsu, 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 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 administration of lactacystin at a dose of 0.1 mg/kg tended to attenuate the ischemia/reperfusion-induced deterioration of renal function, but such effects were partial and observed changes were not statistically significant, except for Pcr. When 1 mg/kg lactacystin was given, all renal function changes induced by the ischemia/reper-

Results

Renal Function after the Ischemia/Reperfusion and Effect of Lactacystin. As shown in Fig. 1, renal function of rats subjected to 45-min ischemia showed a marked deterioration when measured 24 h after the reperfusion. As compared with sham-operated rats, untreated ARF rats showed significant increases in BUN (22.5 ± 0.8 versus 70.1 ± 7.9 mg/dl), Pcr (0.65 ± 0.01 versus 1.82 ± 0.27 mg/dl), urine flow (UF) (33.1 ± 2.8 versus 74.0 ± 6.0 µl/min/kg), FENa (0.57 ± 0.09 versus 1.73 ± 0.38%), and significant decreases in Ccr (5.21 ± 0.42 versus 1.84 ± 0.29 ml/min/kg) and Usom (1426 ± 72 versus 557 ± 52 mOsm/kg). The administration of lactacystin at a dose of 0.1 mg/kg tended to attenuate the ischemia/reperfusion-induced deterioration of renal function, but such effects were partial and observed changes were not statistically significant, except for Pcr.
fusion were significantly and markedly suppressed (BUN, 35.0 ± 1.9 mg/dl; Pcr, 0.90 ± 0.03 mg/dl; Ccr, 3.57 ± 0.41 ml/min/kg; UF, 43.6 ± 4.5 ml/min/kg; FENa, 0.43 ± 0.09%; Uosm, 930 ± 35 mOsm/kg).

**Histological Renal Damage after Ischemia/Reperfusion and Effects of Lactacystin.** Histopathological examination revealed severe lesions in the kidneys of untreated ARF rats (24 h after the 45-min ischemia). These changes were characterized by tubular necrosis in the outer zone outer stripe of medulla, proteinaceous casts in tubuli in the inner zone of medulla, and medullary congestion and hemorrhage in the outer zone inner stripe of medulla. Pretreatment with 0.1 mg/kg lactacystin tended to attenuate the histological damages, but its effects were not statistically significant. Lactacystin at the higher dose (1 mg/kg) prevented the development of all these lesions (Table 1). Typical photographs of each group are shown in Figs. 2 through 4.

**Renal ET-1 Content after the Ischemia/Reperfusion and Effect of Lactacystin.** To evaluate whether the preventive effects of lactacystin on ischemia/reperfusion-induced renal injury could be accompanied by a suppression of the enhanced ET-1 production in the kidney of ischemic ARF rats, renal ET-1 contents at 24 h after the ischemia/reperfusion were determined. As shown in Table 2, renal ET-1 contents were significantly increased in animals exposed to the 45-min ischemia, being about 2-fold over the sham-operated group; however, pretreatment with lactacystin did not suppress the increased ET-1 contents at 24 h after the ischemia/reperfusion, even at the higher dose. Based on recent observations that ET-1 overproduction occurs at an early phase after the ischemia/reperfusion of the kidney (Wilhelm et al., 1999; Kuro et al., 2000), we next measured the renal ET-1 contents at 2 and 6 h after the ischemia/reperfusion (Fig. 5). Renal ET-1 content in untreated ARF increased at 2 h after the reperfusion (untreated ARF, 0.39 ± 0.05 versus sham, 0.18 ± 0.03 ng/g of tissue). This increase was more marked at 6 h after the reperfusion (untreated ARF, 0.69 ± 0.06 versus sham, 0.25 ± 0.03 ng/g of tissue). Lactacystin at 1 mg/kg abolished the elevation of renal ET-1 contents at these early phases (0.19 ± 0.03 and 0.21 ± 0.02 ng/g of tissue, at 2 and 6 h, respectively).

**Discussion**

Many observations indicate that regulated intracellular proteolysis is an important mechanism for controlling key reactions underlying both normal and pathological processes. Most cells contain two major nonlysosomal neutral protein-

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**TABLE 1**

Histopathological changes in ARF rats

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<tr>
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<th>Sham (n = 6)</th>
<th>Untreated ARF (n = 6)</th>
<th>ARF + Lactacystin</th>
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<td>0.1 mg/kg (n = 5)</td>
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<td>Histopathological</td>
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<td>Changes/grade</td>
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<td>Tubular necrosis</td>
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<td>Proteinaceous casts in tubuli</td>
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<td>Medullary congestion</td>
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P < 0.01, compared with untreated ARF.

P < 0.05.
ases in the cytosol. One is calpain, which is known to be a mediator of hypoxic/ischemic injury in brain (Lee et al., 1991) and liver (Bronk and Gores, 1993). This proteinase has also been implicated in hypoxic injury in renal proximal tubules (Edelstein et al., 1996). Thus, we recently examined whether a calpain inhibitor has protective effects on ischemic ARF in animal models; however, results obtained from a previous study (Takaoka et al., 1999) were somewhat unexpected because calpeptin, a potent peptide aldehyde inhibitor of calpain (Tsujinaka et al., 1988), at a dose of 1 mg/kg, did not have a significant protective effect against the deterioration of renal function in ischemic ARF rats. On the other hand, when the effect of PSI (1 mg/kg), as an inhibitor of proteasome that is another cytosolic neutral proteinase, was examined under the same conditions, this inhibitor had preventive effects on the ischemic ARF. These observations implied that the ineffectiveness of calpeptin could be considered a negative control that is required for determining the involvement of proteasome in the effect of a proteasome inhibitor with calpain-inhibiting activity, such as PSI. Therefore, it was reasonable to consider that the preventive effect of PSI on ischemic ARF is not due to the inhibition of calpain but must result from the inhibition of proteasome. In addition, it seemed likely that proteasome occupies an important position in the pathogenesis of ischemic ARF, although we could not exclude the possibility that calpain is involved in the pathogenesis of this type of ARF just because calpeptin did not significantly improve the ischemic ARF. Based on these findings, we noted that proteasome participates in the pathogenesis of ischemic ARF.

In the present study, we used lactacystin, which is a potent and selective proteasome inhibitor that does not affect other proteinases examined so far (Fenteany et al., 1995), to evaluate the involvement of proteasome in the development of ischemic ARF, and obtained evidence in support of our previous notion described above. The administration of lactacystin at a dose of 0.1 mg/kg tended to attenuate the deterioration of renal function induced by ischemia/reperfusion. The preventive effect of a higher dose of lactacystin (1 mg/kg) was potent, and values of some renal function parameters were approximate to those seen in sham-operated control rats. The effectiveness of lactacystin at each dose was similar in extent to that seen with PSI in our previous investigation (Takaoka et al., 1999). These results showed that lactacystin was capable of preventing renal function impairment in rats with ischemia/reperfusion-induced ARF, as well as PSI. Histological renal damage induced by this ischemic ARF was also prevented by treatment with the higher dose of lactacystin. In addition, lactacystin at the higher dose abolished the elevation of renal ET-1 level at an early phase after the reperfusion. These findings indicate that the selective proteasome inhibition with lactacystin improves the renal dysfunction and tissue injury by ischemia/reperfusion, and these effects are accompanied by suppression of renal production of ET-1, a deleterious mediator in the pathogenesis of this type of ARF.

**Fig. 2.** Light microscopy of the outer zone outer stripe of medulla of the kidney treated with vehicle (b), lactacystin 0.1 mg/kg (c), or lactacystin 1 mg/kg (d) 1 day after the ischemia/reperfusion, and the kidney of a sham rat (a). Lactacystin was given intraperitoneally 1 h before the ischemia (45 min). Arrows indicate tubular necrosis (hematoxylin and eosin staining, original magnification, 200×).
Shortly after the discovery of ET, it was suggested that ET might be an important mediator of ARF, because of its intense renal vasoconstrictive properties (Firth et al., 1988). Kon et al. (1989) subsequently noted that intrarenal arterial injection of an ET antibody ameliorated the decreases in renal blood flow and glomerular filtration rate induced by ischemia/reperfusion. Moreover, pharmacological studies using ET receptor antagonists (Mino et al., 1992; Gellai et al., 1995; Birck et al., 1998; Kuro et al., 2000) and ET-converting enzyme inhibitors (Vemulapalli et al., 1993; Matsumura et al., 2000) support the possibility of ET-1 as a causal factor of ischemic ARF. It also has been shown that ET-1 mRNA expression, ET-1 content, and its affinity for ET receptors are elevated in the postischemic kidney (Shibouta et al., 1990; Firth and Ratcliffe, 1992; Nambi et al., 1993; Kuro et al., 2000). A recent study by Wilhelm et al. (1999) indicated that initial ET-1 gene 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 may play 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. In the present study, we also observed that ET-1 content in untreated ARF rats increased significantly 2 h after the reperfusion. This increase was more marked 6 h after the reperfusion, and thereafter, the increased level appeared to decrease gradually but remained higher even at 24 h after the reperfusion, compared with those in sham-operated control animals (Table 2 and Fig. 5). The increases in ET-1 content at 2 and 6 h after the reperfusion were reduced to the sham level by treatment with the higher dose of lactacystin. On the other hand, the elevated renal ET-1 level at 24 h after the reperfusion could not be suppressed by the lactacystin treatment. From these observations, it seems likely that pharmacological actions of lactacystin on the renal ET-1 production in ischemic ARF rats would not be long-lasting, although there is no available information for the pharmacokinetic profile and metabolism of lactacystin in animals. In addition, it is conceivable that the suppressive effect of lactacystin on the renal ET-1 production enhanced in an early phase after reperfusion would attenuate actions of ET-1 on the kidney in ischemic ARF rats and, eventually, ameliorate the renal function impairment and tissue injury that are observed 24 h after the reperfusion.

It is difficult to clarify the regulatory mechanism of the renal ET-1 production by proteasome in an in vivo study. Corder et al. (1997) showed that peptide aldehyde inhibitors of proteasome, such as calpain inhibitor-I and MG115, blocked tumor necrosis factor (TNF)-α-stimulated ET-1 synthesis in cultured bovine aortic endothelial cells. It is known that treatment of endothelial cells with a variety of stimuli including TNF-α results in the rapid activation of NF-κB

Fig. 3. Light microscopy of the inner zone of medulla of the kidney treated with vehicle (b), lactacystin 0.1 mg/kg (c), or lactacystin 1 mg/kg (d) 1 day after the ischemia/reperfusion, and the kidney of a sham rat (a). Lactacystin was given intraperitoneally 1 h before the ischemia (45 min). Arrows indicate proteinaceous cast in tubuli (hematoxylin and eosin staining, original magnification, 200×).
(Collins, 1993), which is found in the cytoplasm of most cells as an inactive complex bound to an inhibitory protein, IκB, through the phosphorylation of IκB, and its subsequent proteolytic degradation by the proteasome-dependent proteolytic pathway (Palombella et al., 1994). It remains to be determined if NF-κB regulates ET-1 gene expression; however, database analysis of human ET-1 gene reveals consensus recognition motifs for NF-κB binding in the 5'-flanking sequence from the transcription start site. This led us to examine the precise mechanism of proteasome inhibitors on the ET-1 production, using cultured vascular endothelial cells. We have now obtained findings that in cultured porcine aortic endothelial cells, both PSI and lactacystin partially suppress basal ET-1 mRNA expression and completely suppress TNF-α-induced expression by inhibiting the activation of NF-κB by proteasome (M. Ohkita, M. Takaoka, Y. Kobayashi, E. Itoh, H. Uemachi, and Y. Matsumura, unpublished data). In the present study, we found that lactacystin attenuated ischemia/reperfusion-induced ET-1 content in the kidney. Taken together, one possible mechanism whereby proteasome inhibition ameliorates renal dysfunction and degeneration in ischemic ARF is attributed to the

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aP < 0.01, compared with sham rats.
inhibition of enhanced expression of renal ET-1 via NF-κB activation in an early phase after the reperfusion.

Lactacystin is a useful tool for clarifying physiological and pathophysiological roles of proteasome. Dick et al. (1996) reported that lactacystin enters cells and inhibits proteasome, as a β-lactone derivative. Recently, a novel, small-molecular-weight analog of lactacystin, clasto-lactacystin β-lactone (called PS519), was synthesized (Soucy et al., 1999) and shown to elicit cardioprotective effects following ischemia/reperfusion in isolated perfused rat heart (Campbell et al., 1999) and to exhibit cerebroprotective properties in a rat model of focal cerebral ischemia (Phillips et al., 2000). Taken together with the present data showing renoprotective effects of lactacystin in ischemic ARF rats, the use of selective proteasome inhibitors may be a novel approach to the treatment of diseases in which etiology is dependent on ischemia and reperfusion. Furthermore, findings from the present study suggest that selective proteasome inhibition may also be a pertinent treatment of cardiovascular diseases such as neointimal thickening following balloon injury and atherosclerosis that results from aberrant ET-1 production or NF-κB activation (Brand et al., 1996; Wang et al., 1996; Cereck et al., 1997).

In conclusion, our results clearly indicate the effectiveness of lactacystin and the crucial role of a proteasome-dependent proteolytic pathway in the pathogenesis of ischemic ARF, possibly through the enhancement of ET-1 production in renal injury.

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