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Enhancement of protein kinase C activity and chemiluminescence intensity in mitochondria isolated from the kidney cortex of rats treated with cephaloridine

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Abstract

The development of nephrotoxicity induced by cephaloridine (CER) has been reported to be due to reactive oxygen species (ROS). Protein kinase C (PKC) has been suggested to modulate the generation of ROS. We investigated the possible participation of ROS generation assessed by chemiluminescence (CL) and PKC activity in rat kidney cortical mitochondria in the development of CER-induced nephrotoxicity. We first evaluated the magnitude of the nephrotoxic damage caused by CER in rats. The plasma parameters and ultrastructural morphology changes were increased markedly 24 hr after the treatment of rats with CER. We demonstrated that the treatment of rats with CER clearly evoked not only enhancement of *Cypridina* luciferin analog (CLA)-dependent CL intensity, but also the activation of PKC in mitochondria isolated from the kidney cortex of rats 1.5 and 3.5 hr after injection of the drug. These changes were detected in advance of those observed in plasma and by electron microscopy. The increase in CLA-dependent CL intensity detected in the kidney cortical mitochondria 1.5 and 3.5 hr after injection of CER was inhibited completely by the addition of superoxide dismutase, suggesting the generation of superoxide anion in these mitochondria during the early stages of CER-induced nephrotoxicity. These results suggest that the activation of PKC and the enhancement of superoxide anion generation in kidney cortical mitochondria precede the increases in plasma parameters and the electron micrographic changes indicative of renal dysfunction in rats treated with CER. Additionally, they suggest a possible relationship between PKC activation in mitochondria and free radical-induced CER nephrotoxicity in rats.

Keywords: Cephaloridine; Nephrotoxicity; Reactive oxygen species; Chemiluminescence; Protein kinase C; Kidney cortical mitochondria

1. Introduction

CER, a cephalosporin antibiotic, has been reported to induce acute renal failure as a side-effect in humans as well as in experimental animals [1–3]. CER administration results in renal damage characterized by acute proximal tubular necrosis, most notably of the S2 segment of the tubules [4], where CER is transported by the renal basolateral transport system for organic anions from blood to proximal tubular cells. However, CER is limited to movement into the tubular lumen leading to its high concentration within proximal tubular cells, which are critical for the development of nephrotoxicity [5–7]. It has been proposed that the development of nephrotoxicity induced by CER may be due to free radicals such as ROS [8–12]. ROS-induced lipid peroxidation appears to be the most likely mechanism of renal injury caused by CER [13,14]. We previously demonstrated that free radical-mediated injury induced by CER is modulated by phorbol myristate acetate, a PKC activator, in rat renal cortical slices, suggesting that PKC activation enhances CER-induced nephrotoxicity [15]. PKC has been suggested to be important for mediating cell injury and for modulating lipid peroxidation in hepatocytes exposed to ROS [16]. Mitochondria have been reported to be one source of free radical generation such as ROS in renal injury caused by some nephrotoxicants [17,18]. Oxygen radical generation is assessed by CL, which has been used for the detection of ROS in activated leukocytes [19,20], the myeloperoxidase system *in vitro* [21], the lungs of rats

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Abbreviations: BUN, blood urea nitrogen; CER, cephaloridine; CL, chemiluminescence; CLA, *Cypridina* luciferin analog; PKC, protein kinase C; ROS, reactive oxygen species.

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with necrotized pancreatitis [22], and the stomachs of rats after ischemia/reperfusion [23]. The aim of the present study was to examine the possible participation of ROS generation, assessed by CL and PKC activity in kidney cortical mitochondria, in the development of CER-induced nephrotoxicity.

2. Materials and methods

2.1. Chemicals

CER and superoxide dismutase were purchased from the Sigma Chemical Co. A *Cypridina* luciferin analog, 2-methyl-6-phenyl-3,7-dihydro-imidazo[1,2-*a*]pyrazin-3-one, which reacts with O_2^- or $^1O_2$ to emit light in the visible region [24,25], was obtained from the Tokyo Kasei Industries Co. [γ-^{32}P]ATP was obtained from Amersham Pharmacia Biotech UK Ltd. All other chemicals used were of the highest purity available (Wako Pure Chemical Industries Ltd.).

2.2. Experimental animals

Male Sprague–Dawley rats weighing 180–250 g were used, and were allowed free access to tap water and a standard laboratory diet. They were given 1.2 g/kg of CER dissolved in isotonic saline by intravenous injection. Control animals were administered the same volume (1.5 mL/kg) of isotonic saline. The rats were killed under pentobarbital anesthesia (50 mg/kg, i.p.), and their kidneys were removed 1.5, 3.5, and 24 hr after the injections.

2.3. Renal functional parameters

Blood was collected from the abdominal aorta at the times indicated. Plasma creatinine and urea nitrogen concentrations were measured using a spectrophotometer.

2.4. Electron microscopy

Transverse sections of the kidney cortex at 1.5, 3.5, and 24 hr after treatment with CER were minced into 1-mm cubes. The samples were fixed in 2.5% glutaraldehyde and 1% formaldehyde in 0.1 M phosphate buffer, pH 7.4, and refrigerated for up to 24 hr. Samples were postfixed in 1% osmium tetroxide in 0.1 M phosphate buffer, dehydrated through a graded series of ethanol and propylene oxide, and then embedded in epoxy resin. Semithin sections of approximately 1 μm thickness were cut with glass knives and stained with toluidine blue. After areas of interest were identified, ultrathin sections were cut with diamond knives and stained with 1% uranyl acetate for 20 min and with 1% lead citrate for 2 min. Electron microscopic images were obtained using an HITACHI H-600 transmission electron microscope.

2.5. Preparation of mitochondria

Mitochondria were prepared from the kidney cortex of rats treated with CER by a modification of the method [26,27] of Greenawalt and Schnaitman [28]. The kidneys were homogenized at 0° in a glass homogenizer and centrifuged at 850 *g* for 10 min at 4° in solution A, which consisted of a 0.25 M sucrose and 3 mM $MgCl_2$ solution, containing 25 mM β-glycerophosphate, 1 mM dithiothreitol, 1 mM orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/mL of aprotinin. The resultant supernatant fluid was recentrifuged at 850 *g* for 10 min at 4°, yielding a second supernatant fraction which was centrifuged at 10,000 *g* for 10 min at 4°. This supernatant (S1) was used for the preparation of cytosolic and microsomal fractions. The resultant pellets were resuspended in solution A, and recentrifuged at 10,000 *g* for 10 min at 4°. The precipitate was resuspended in solution A to be used as the mitochondria fractions. A part of the mitochondrial pellets were disrupted in lysis buffer at 4° for 30 min and then centrifuged at 15,000 *g* for 15 min at 4° for the determination of mitochondrial proteins in the supernatant.

2.6. Preparation of cytosolic and microsomal fractions

The microsomal fraction was prepared as follows: the supernatants (S1) obtained by centrifugation at 10,000 *g* for 10 min at 4° were then centrifuged at 100,000 *g* for 1 hr at 4°. The resultant supernatant was used as the soluble cytosolic fraction. The pellet was suspended in solution A to be used as the microsomal fraction.

2.7. Protein determination

Protein concentration was determined using a protein assay kit with bovine serum albumin as a standard (Pierce).

2.8. Measurement of active oxygen radical generation in mitochondria and microsomes

The mitochondria and microsomes were suspended in Hanks'-buffered saline solution, pH 7.4. ROS were measured as CLA-dependent CL, which is, in turn, dependent upon O_2^- generation, as described previously [24,25]. CLA was added to the sample tube containing the mitochondria or microsomal fraction in Hanks'-buffered saline. The CL of the mitochondrial and microsomal fractions was measured using a Luminescence Reader (CAF-110). The CLA-dependent CL was assessed with the peak value of CL expressed as counts/mg protein.

2.9. PKC assay

PKC enzyme activity was measured as ^{32}P incorporation from [γ-^{32}P]ATP into a peptide in the presence of Ca^{2+}, using a PKC enzyme assay system kit (Amersham

Pharmacia Biotech). Prior to the PKC assay, the microsomes and mitochondria were dissolved in a Triton X-100/DMSO mixture, incubated in ice-cold water for 60 min, and then centrifuged at 5000 *g* for 15 min at 4° to remove undissolved material.

The reaction mixture (pH 7.5) in a polypropylene tube consisted of 50 mM Tris–HCl, 12 mM magnesium chloride, 3 mM calcium acetate, 225 μM peptide, 0.2 mM ATP, and 0.2 μCi [γ-^{32}P]ATP. The reaction was started by the addition of the cytosolic, microsomal, and mitochondrial fractions, was incubated at 37° for 15 min, and was stopped by the addition of 300 mM orthophosphoric acid. The terminated reaction mixture was transferred to peptide-binding paper discs. The peptide-binding paper discs were washed twice with 75 mM H_3PO_4 for 5 min, and added to 10 mL of liquid scintillation fluid. The results are expressed in picomoles phosphate transferred/min/mg protein.

2.10. Statistical analysis

Results are expressed as mean ± SEM. The significance of the differences between the groups was analyzed by Scheffe's *F* test after analysis of variance. *P* values less than 0.05 were regarded as statistically significant.

3. Results

3.1. Plasma parameters and ultrastructural morphology changes indicative of renal dysfunction after CER treatment in rats

Rats were given a single intravenous injection of 1.2 g/kg of CER and were evaluated for nephrotoxicity. BUN was increased markedly 24 hr after treatment, but was not changed at 1.5 and 3.5 hr (Fig. 1A). Plasma levels of creatinine also increased 24 hr after treatment with CER but not at 1.5 and 3.5 hr (Fig. 1B). The change in the BUN level after treatment with CER closely paralleled that of creatinine.

The CER-induced pathology in the rat kidneys was verified by electron microscopy. There were no pathological changes in the proximal tubules of rats treated with CER at 1.5 hr (Fig. 2B). However, electron micrographs showed a marked dilation of the vacuolar apparatus, swollen mitochondria, and loss of the brush border in the proximal tubules 24 hr after exposure to CER (Fig. 2C).

3.2. Changes in CLA-dependent chemiluminescence

The maximum increase in CLA-dependent CL intensity was detected in mitochondria prepared from the kidney cortex of rats 1.5 hr after injection of CER. CLA-dependent CL intensity in mitochondria was decreased by 3.5 hr after injection of CER, but the CL intensity was significantly higher than that in mitochondria from control rats (Fig. 3). The CL intensity in kidney cortical mitochondria from rats 24 hr after CER injection was at a level similar to that in control mitochondria. Such increases in CLA-dependent CL intensity in mitochondria were reduced to the control level in the presence of superoxide dismutase (Fig. 3). There was no significant difference in the CLA-dependent CL intensity in microsomes prepared from the kidney cortex of rats treated with CER (Fig. 4).

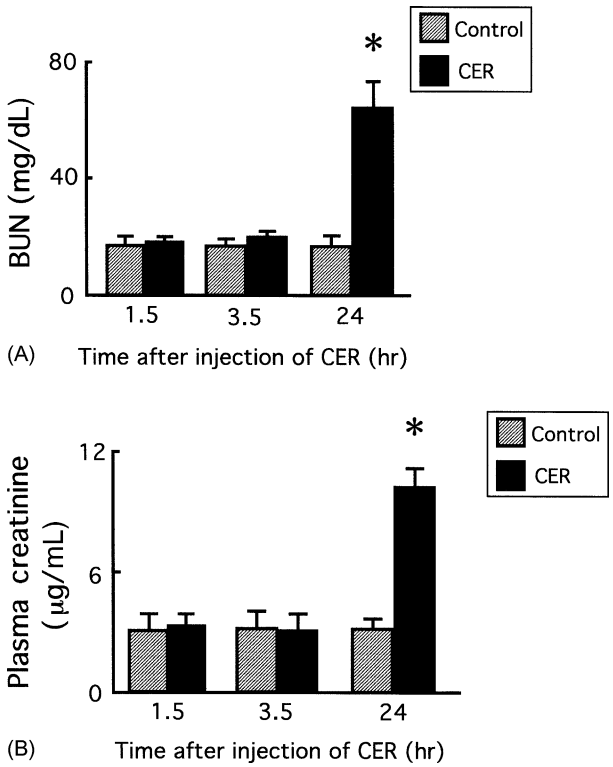

Fig. 1. Changes in blood urea nitrogen (BUN) (A) and plasma creatinine (B) in rats treated with CER (1.2 g/kg, i.v.). Each value represents the mean ± SEM of five experiments. Key: (*) $P < 0.001$, compared with the respective control.

3.3. PKC activity

Activation of PKC was detected in the mitochondria 1.5 and 3.5 hr after injection of CER (Fig. 5), whereas there was no change in the microsomal fraction from the kidney cortex of rats treated with CER (Fig. 6). The cytosolic PKC activity was reduced significantly at 1.5 and 3.5 hr after treatment of rats with CER (Fig. 7). There was no change in the cytosolic, microsomal, or mitochondrial PKC activity 24 hr after treatment of rats with CER (Figs. 5–7).

4. Discussion

In the present study, a CER dose of 1.2 g/kg, given intravenously, was selected as the dose needed to affect nephrotoxic indices in plasma and induce ultrastructural

changes indicative of renal damage 24 hr after treatment of the rats with the antibiotic. We demonstrated that CER treatment clearly evoked not only the enhancement of CLA-dependent CL intensity but also the activation of PKC in kidney cortical mitochondria isolated from rats at 1.5 hr after the drug injection, indicating such changes in advance of those in plasma parameters and on electron micrographs. The significant increase in CLA-dependent CL intensity detected in the kidney cortical mitochondria at 1.5 and 3.5 hr after injection of CER was inhibited completely by the addition of superoxide dismutase. This suggests the generation of superoxide anions in the mitochondria from the kidney cortex of rats treated with CER during the early stage of the development of CER nephrotoxicity. Cojocel *et al*. [14] demonstrated that CER is reduced by microsomes and yields an electron to oxygen to generate the superoxide anion. The present results do not disprove CER-induced generation of ROS in microsomes. Under the current experimental conditions, the increase in CLA-dependent CL intensity was detected in mitochondria in the presence of succinic acid and oxygen, possibly as a source of superoxide anion. These findings suggest that the

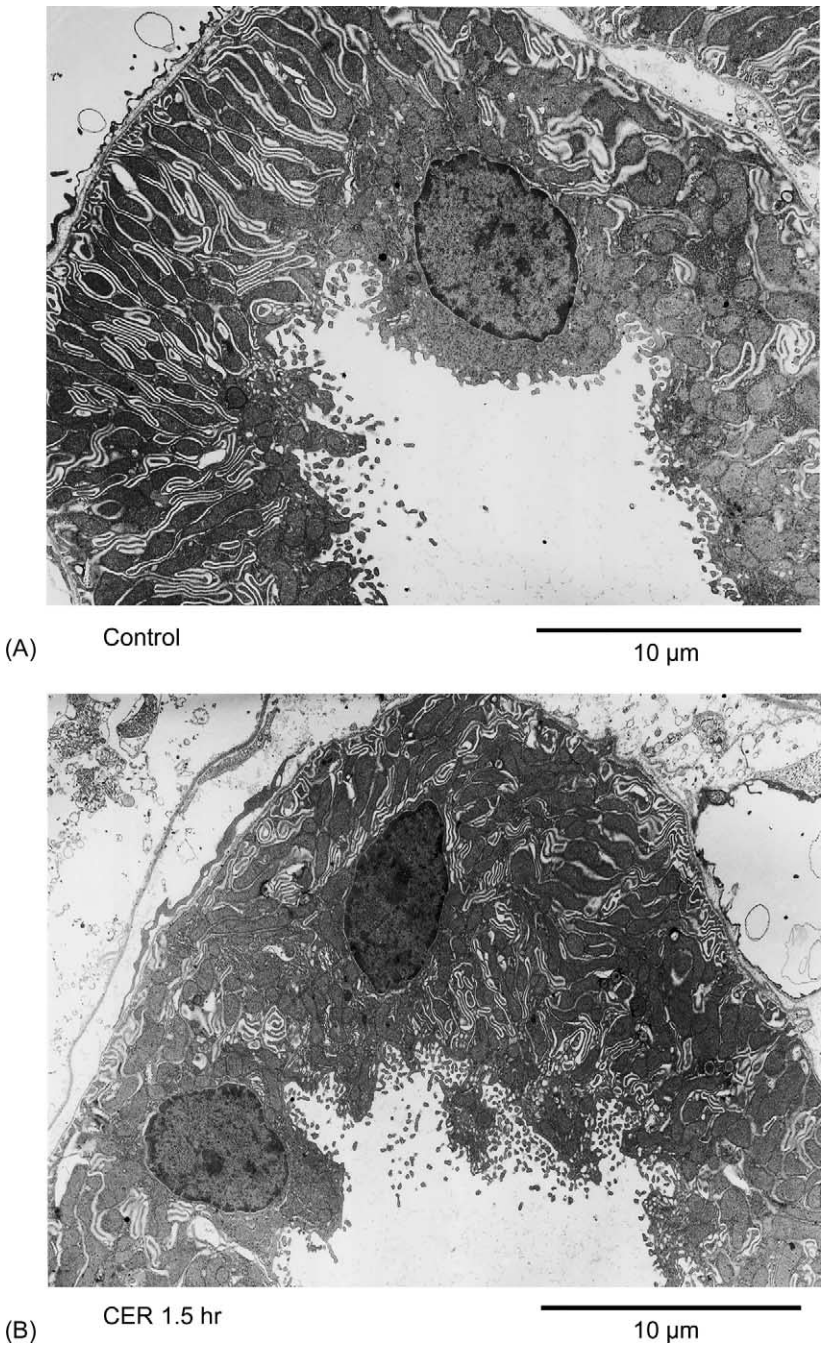

Fig. 2. Electron microscopic images of renal proximal tubules of rats treated with vehicle for 24 hr (A) and cephaloridine (CER, 1.2 g/kg) for 1.5 hr (B) and for 24 hr (C). The arrows indicate, from left to right, the loss of the brush border, vacuolar apparatus, and swollen mitochondria. Scale bar, 10 μm.

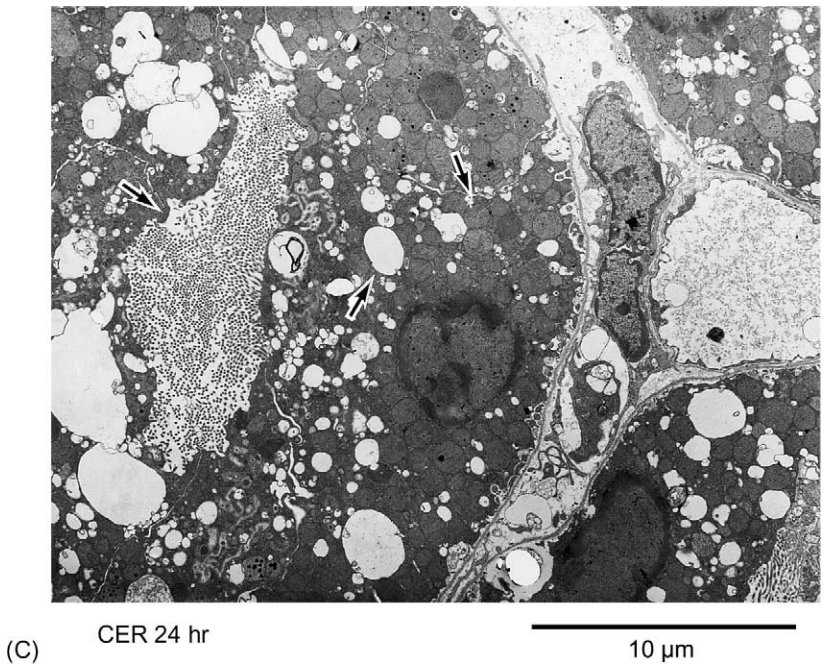

Fig. 2. (*Continued*).

superoxide anion is probably produced through the mitochondrial respiratory chain. It is widely assumed that the increased electron flow through the mitochondrial electron transport chain leads to an increased rate of ROS production [29,30]. The mitochondrial respiratory chain could also be a potential source of ROS in tissues, such as the kidneys, liver, and muscles [31–33]. If mitochondrial production of superoxide anion contributes to CER-induced free radical generation, the mitochondria could be a primary target of CER-induced oxidative stress.

PKC activity was increased significantly in mitochondria and decreased in cytosol at 1.5 and 3.5 hr after treatment of rats with CER (Figs. 5 and 7). These results suggest that CER probably induced translocation of PKC from the cytosol into the mitochondria. It has been demonstrated that the phorbol ester-induced translocation of PKCδ from cytosol to mitochondria occurs in human leukemia cells [34]. Regarding PKC isoenzymes, there is evidence that various PKC isoenzymes are expressed in the kidney. It has been reported that PKC isoenzymes α, β, δ, ε, and ζ are expressed in rat kidney cortex [35]. Pfaff *et al*. [36] have demonstrated the presence of PKC α, β1, and β2 in rat kidney cortex. It remains to be clarified which PKC isoenzymes are involved in the development of CER

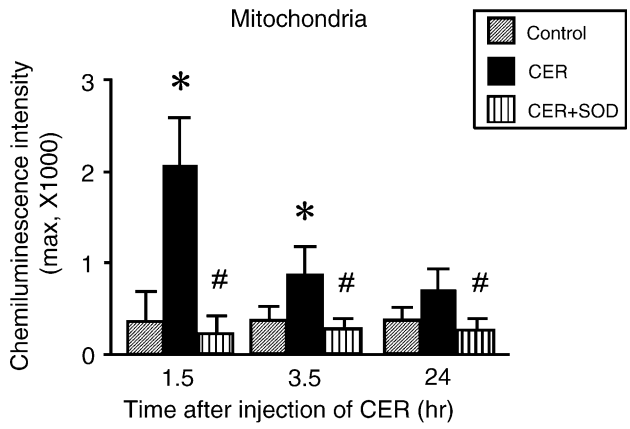

Fig. 3. Effect of cephaloridine (CER) on CLA-dependent chemiluminescence in mitochondria prepared from kidney cortex of rats treated with CER (1.2 g/kg, i.v.). After the addition of the mitochondrial suspension (0.1 mg/mL), CLA-dependent CL was measured in the presence or absence of superoxide dismutase (SOD, 1.6 IU/mL). Each value represents the mean ± SEM of five experiments. Key: (*) $P < 0.01$, compared with the respective control, and (#) $P < 0.01$, compared with CER.

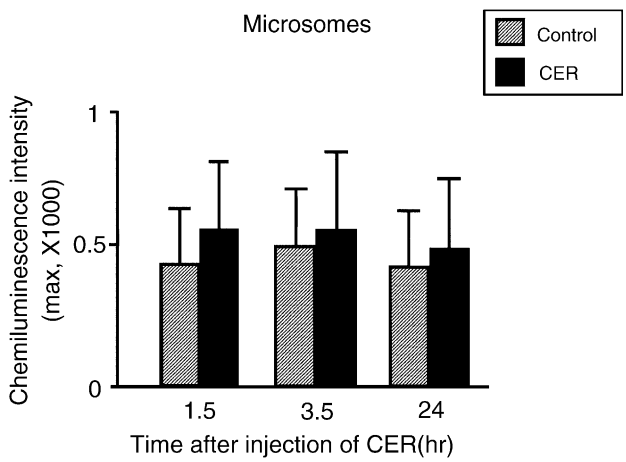

Fig. 4. Effect of cephaloridine (CER) on CLA-dependent chemiluminescence in microsomes prepared from the kidney cortex of rats treated with CER (1.2 g/kg, i.v.). Each value represents the mean ± SEM of five experiments. There were no significant differences between the control and the CER group.

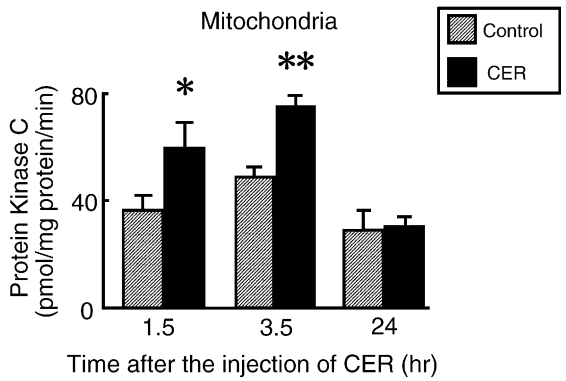

Fig. 5. Effect of cephaloridine (CER) on the activity of protein kinase C in mitochondria prepared from kidney cortex of rats treated with CER (1.2 g/kg, i.v.). Each value represents the mean ± SEM of five experiments. Key: (*) $P < 0.05$, and (**) $P < 0.01$ compared with the respective control.

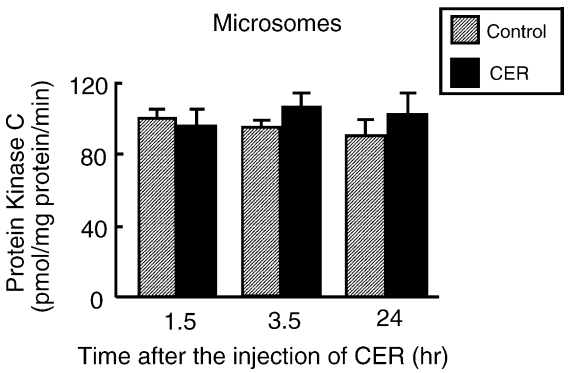

Fig. 6. Effect of cephaloridine (CER) on the activity of protein kinase C in microsomes prepared from kidney cortex of rats treated with CER (1.2 g/kg, i.v.). Each value represents the mean ± SEM of five experiments. There was no significant difference between the control and CER.

nephrotoxicity. The relationship between the activation of PKC and the enhancement of CL intensity should be solved in future studies. Our preliminary experiments have shown that the administration of H-7, a PKC inhibitor, prevents ROS generation in the mitochondria and the nephrotoxicity induced by CER, suggesting the participation of PKC in

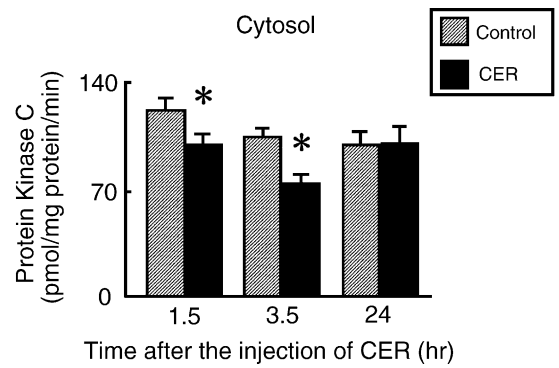

Fig. 7. Effect of cephaloridine (CER) on the activity of protein kinase C in cytosol prepared from kidney cortex of rats treated with CER (1.2 g/kg, i.v.). Each value represents the mean ± SEM of five experiments. Key: (*) $P < 0.01$, compared with the respective control.

ROS generation and nephrotoxicity caused by CER. The most noteworthy point is the correlation between the intracellular sites of PKC activation and ROS generation, that is, a common intracellular site affected by CER was the mitochondria. Recent studies have revealed that phorbol myristate acetate (PMA), an activator of PKC, can induce superoxide generation in the immune system, especially neutrophils [37,38], emphasizing that PMA-induced superoxide production is dependent upon the activation of PKC. The present findings, considered together with those mentioned above, indicate that the early activation of mitochondrial PKC is probably reno-toxic, and may have a significant role in superoxide anion generation in the kidney cortical mitochondria of rats treated with CER.

In conclusion, the activation of PKC and the enhancement of superoxide anion generation in kidney cortical mitochondria preceded the increases in the plasma parameters and the electron micrographic changes indicative of renal dysfunction in rats treated with CER, suggesting the possible relationship of PKC activation in mitochondria to free radical-induced CER nephrotoxicity in rats.

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