Modulation by Cyclic AMP and Phorbol Myristate Acetate of Cephaloridine-Induced Injury in Rat Renal Cortical Slices

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ABSTRACT—Intracellular signaling pathways of cAMP and protein kinase C (PKC) have been suggested to modulate the generation of free radicals. We investigated the effects of cAMP and phorbol myristate acetate (PMA), a PKC activator, on cephaloridine (CER)-induced renal cell injury, which has been reported to be due to the generation of free radicals. Incubation of rat renal cortical slices with CER resulted in increases in lipid peroxidation and lactate dehydrogenase (LDH) release and decreases in gluconeogenesis and p-aminohippurate (PAH) accumulation in rat renal cortical slices, suggesting free radical-induced injury in slices exposed to CER. A derivative of cAMP ameliorated not only the increase in lipid peroxidation but also the renal cell damage induced by CER. This amelioration by a cAMP derivative of lipid peroxidation and renal cell damage caused by CER was blocked by KT 5720, a protein kinase A (PKA) inhibitor. Lipid peroxidation and the indices of cell injury were increased by PMA. PMA also enhanced CER-induced lipid peroxidation and cell damage in the slices. This enhancement by PMA of CER-induced injury was blocked by H-7, a PKC inhibitor. These results indicated that intracellular signaling pathways of cAMP and PKC modulate free radical-mediated nephrotoxicity induced by CER.

Keywords: Cephaloridine, Nephrotoxicity, Free radical, cAMP, Phorbol myristate acetate

Cephaloridine (CER), a first-generation cephalosporin antibiotic, causes acute renal failure as a side effect in humans and experimental animals (1 – 3). CER-induced nephrotoxicity is characterized by necrosis of proximal tubules, most notably of the S2 segment of the tubules (4). CER is transported by an organic anion transport system from blood to proximal tubular cells. However, CER is restricted in its capacity of moving into the tubular fluid, resulting in its high concentration within proximal tubular cells, critical for the development of nephrotoxicity (5 – 7). It has been reported that the development of nephrotoxicity induced by CER has been suggested to be due to free radicals such as reactive oxygen species (ROS) (8 – 15). Protein kinase C (PKC) has been suggested to be important for mediating cell injury and for modulating lipid peroxidation in hepatocytes exposed to ROS (16). Previous studies have indicated that a phosphodiesterase inhibitor and dibutyryl cAMP ameliorate the cell injury induced by CER in a cultured renal epithelial cell line, LLC-PK1, derived from porcine kidney (17). It has also been demonstrated that cAMP inhibits the production of ROS stimulated by phorbol 12-myristate 13-acetate (PMA), a PKC activator, in rat glomeruli (18). Little is known about the participation of PKC in the development of CER-induced nephrotoxicity. Although the amelioration of CER-induced injury by cAMP has been demonstrated using established renal epithelial cells as described above, these cells may have differentiated and may differ from the renal tissue of origin (19). Present experiments were performed to study the effects of cAMP and PMA on CER-induced injury, which was evaluated by the an in vitro technique using slices freshly prepared from the rat renal cortex.

MATERIALS AND METHODS

Chemicals
Cephaloridine, 8-(4-chlorophenylthio)-cAMP sodium salt (CTcAMP), 1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride (H-7) and PMA were purchased from Sigma Chemical Co. (St. Louis, MO, USA). KT 5720 ((8R,9S,11S)-(1)-9-hydroxy-9-hexoxyxcarbonyl-8-methyl-2,3,9,10-tetrahydro-8,11-epoxy-1H,8H,11H-2,7b,11a-triazadibenzo[a,g]cycloocta[cd]triden-1-one) was from Calbiochem-Novabiochem Co. (La Jolla, CA, USA). All other chemicals used were of the highest purity available (Wako
Preparation and incubation of renal cortical slices

Male Sprague-Dawley rats weighing 220 – 270 g were used. The animals were allowed free access to tap water and to a standard laboratory diet. Rats were sacrificed under pentobarbital anesthesia (50 mg/kg, i.p.), and their kidneys were rapidly removed, decapsulated and placed in ice-cold isotonic saline medium. Renal cortical slices were prepared freehand using a razor blade on an ice-cold petri dish and were placed in ice-cold medium containing 134 mM NaCl, 5.9 mM KCl, 1.5 mM CaCl₂, 1.2 mM MgCl₂, 11.5 mM glucose and 5.8 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), titrated with NaOH at pH 7.4 as already described previously (20). The slices were transferred to flasks containing the same medium. Its composition was identical to the medium. CER, H-7, a PKC inhibitor, PMA, CTCAMP and KT 5720 were added to the incubation medium. The slices were incubated in the medium at 37°C with a gas phase of 100% oxygen. The slices were gently removed from the incubation medium 120 min after the first incubation, blotted to filter paper and transferred immediately to the incubation medium without CER and other drugs, in which the slices were incubated again at 37°C with a gas phase of 100% oxygen for 90 min. After the second incubation, the slices and medium were analyzed for lipid peroxidation, lactate dehydrogenase (LDH) release, gluconeogenesis and p-aminohippurate (PAH) accumulation as described below.

Determination of lipid peroxidation and LDH release

Lipid peroxidation was estimated by measuring the concentration of thiobarbituric acid-reactive substances (TBARS) in the slices and medium according to the method of Buege and Aust (21). Briefly, slices were homogenized in ice-cold 0.15 M KCl and 0.02 M Tris-HCl buffer, pH 7.4. Lipid peroxidation in the homogenate and in the incubation medium, centrifuged at 1,500 × g for 10 min, was measured by assaying the absorbance at 535 nm after the mixtures of samples and thiobarbituric acid were heated in boiling water and then neutralized. The total level of TBARS in the slices and medium was expressed as nmol per g wet weight of slices incubated. LDH activity in the incubation medium released from the slices was determined using a commercial kit (Wako Pure Chemical Industries, Ltd.).

Determination of gluconeogenesis and PAH accumulation

In the second incubation, the slices were incubated in the medium with 10 mM pyruvate instead of 11.5 mM glucose for determination of gluconeogenesis. After removing the slices, the medium was centrifuged at 1,500 × g for 10 min, and the supernatant fraction was used for determination of glucose released from the slices with a commercial kit (Wako Pure Chemical Industries, Ltd.).

The slices were incubated in the medium with 0.074 mM PAH for determination of PAH accumulation. After incubation, the slices were blotted on filter paper, weighed and homogenized with 10% trichloroacetic acid (TCA). Aliquots of samples obtained by centrifugation of the homogenate and medium, which were also treated with 10% TCA, were analyzed to determine the concentration of PAH by the method of Bratton and Marshall (22). PAH accumulation in the slices was expressed as the ratio of the level of PAH in slices (S) to medium (M).

Statistical analyses

Results are expressed as means ± S.E.M. The significance of the difference 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.

RESULTS

Effects of CER on lipid peroxidation, LDH release, gluconeogenesis and PAH accumulation in rat renal cortical slices

We examined the effects of different CER concentrations on lipid peroxidation and the indices of injury in the slices. The level of lipid peroxidation was measured indirectly to evaluate free radical generation; and LDH release, gluconeogenesis and PAH accumulation in the slices were assessed as indices of cell injury induced by CER. CER caused not only an increase in lipid peroxidation level but also an increase in LDH leakage and the decreases in gluconeogenesis and PAH accumulation in renal cortical slices in a concentration-dependent manner (Fig. 1). The results obtained here of CER-induced generation of free radicals causing development of renal cell injury were in good agreement with the findings reported by Kuo et al. (8).

Effects of cAMP on CER- induced renal cell injury

Figure 2 shows the effects of a cAMP derivative capable of permeating the cell membrane and a cAMP-dependent protein kinase (PKA) inhibitor on lipid peroxidation level and the indices of cell injury induced by CER. CER-induced increases in lipid peroxidation level and LDH leakage in the slices were lessened by CTCAMP, a cAMP derivative (Fig. 2: A and B). CER-induced decreases in gluconeogenesis and PAH accumulation in the slices were also lessened by CTCAMP (Fig. 2: C and D). This protective effect of CTCAMP on the indices of cell injury in renal cortical slices exposed to CER was blocked by KT 5720, a PKA inhibitor (23, 24).
Effects of PMA on CER-induced renal cell injury

The involvement of another major signaling pathway, PKC, in lipid peroxidation and cell injury in the slices was examined. PMA, a PKC activator, at a concentration of 12.5 μM had no effect on the level of lipid peroxidation, LDH leakage, gluconeogenesis or PAH accumulation in renal cortical slices (Fig. 3). However, treatment of the slices with 25 μM PMA caused not only increases in lipid peroxidation and LDH leakage but also decreases in gluconeogenesis and PAH accumulation in the slices. Such
changes induced by 25 μM PMA were completely blocked by H-7, a PKC inhibitor (25, 26) (data not shown).

We performed an experiment to determine whether a low concentration of PMA, which itself had no effect on cellular indices in the slices, affected CER-induced renal cell injury. As shown in Fig. 4, PMA at a concentration of 12.5 μM enhanced CER-induced increases in lipid peroxidation level and LDH leakage. CER-induced decreases in gluconeogenesis and PAH accumulation in renal cortical slices were also enhanced by 12.5 μM PMA. Such en-
hancement by PMA of lipid peroxidation level and the indices of injury in renal cortical slices exposed to CER was completely blocked by H-7.

DISCUSSION

The present results indicated that CER-induced lipid peroxidation and cellular injury in renal cortical slices were modified by cAMP and PMA. It has been suggested that free radicals contribute to the development of CER-induced nephrotoxicity (8–15). Our results also suggested that CER generates free radicals, resulting in oxidative damage in renal cortical slices. Miyano et al. reported that a dibutyryl derivative of cAMP inhibited the production of oxygen radicals stimulated by PMA (18). This derivative of cAMP has also been demonstrated to reduce the development of renal ischemia-reperfusion injury in which oxygen radicals have been suggested to be potentially harmful factors (27). It has also been suggested that adenosine protects against free radical-induced nephrotoxicity by increasing the intracellular cAMP level via adenosine receptors (28, 29). We demonstrated previously that a phosphodiesterase inhibitor and dibutyryl cAMP protected against free radical-induced injury in an established renal epithelial cell line, LLC-PK1, exposed to CER (17). In the present study, we demonstrated that a derivative of cAMP ameliorated CER-induced lipid peroxidation and cell injury in the slices and that the effect was completely abolished by a PKA inhibitor, KT 5720, suggesting a preventive effect of PKA activation on free radical generation in renal cortical slices. However, other effects of PKA activation on CER-induced lipid peroxidation are not denied, since there still is a possibility of PKA activation to enhance the cellular ability of protection against free radical-induced injury. PMA at a concentration of 25 μM itself caused increases in lipid peroxidation level and LDH leakage and decreases in gluconeogenesis and PAH accumulation in the slices. This injury by 25 μM PMA was prevented by H-7 (data not shown). PMA, a PKC activator, at a low concentration of 12.5 μM itself did not affect any indices measured in the slices. However, such a low concentration of PMA enhanced CER-induced damage in the slices, and this enhancement induced by PMA in the presence of CER was completely blocked by H-7, a PKC inhibitor. Activation of PKC has also been suggested to increase free radical generation in various cells and to be important for mediating cell injury (16, 30). The present results together with those reported in previously suggested that PKC activation enhances free radical-induced injury in renal cortical slices exposed to CER. Goldstein et al. have reported that antioxidant reduces CER-induced lipid peroxidation, but does not reverse CER-induced decrease of gluconeogenesis (10). We demonstrated that cAMP inhibited CER-induced decrease of gluconeogenesis. It has been suggested that cAMP stimulates the gene expression of phosphoenolpyruvate carboxykinase (PEPCK), which catalyzes the rate-limiting step of gluconeogenesis (31). The present study together with that given in this previous communication may indicate that cAMP inhibits CER-induced decrease of gluconeogenesis through the increase in PEPCK activity. We also demonstrated that 25 μM PMA caused not only the increase in lipid peroxidation but also the decrease in gluconeogenesis. It has been reported that the increase in lipid peroxidation damages enzymes and proteins (32, 33). Thus, the PMA-induced decrease in gluconeogenesis has been suggested to be due to the increase in lipid peroxidation. Some other nephrotoxic drugs have been reported to cause oxidative damage in renal cells (34–40). Further experiments must be performed to clarify the roles of intracellular signaling pathways in free radical-induced renal damage associated with nephrotoxic drugs.

In conclusion, we have shown that the cAMP pathway ameliorates CER-induced renal damage, while PKC activation enhances CER-induced renal injury. It is possible that CER-induced injury of free radicals is modulated by activation of PKC or elevation of intracellular cAMP level caused by activation of each related receptor in renal cortical cells.

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cAMP and PMA on CER-Induced Renal Injury

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