Amelioration by cAMP of Cephaloridine-Induced Injury in the Porcine Kidney Cell Line LLC-PK₁

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ABSTRACT—We investigated the effects of a phosphodiesterase inhibitor and dibutyryl cAMP (dBcAMP) on the cell injury induced by cephaloridine (CER) in an established renal epithelial cell LLC-PK₁. CER increased the leakage of lactate dehydrogenase (LDH) from LLC-PK₁ cells to the medium and the level of lipid peroxidation in the cells. 3-Isobutyl-1-methylxanthine, a phosphodiesterase inhibitor, increased cAMP content in LLC-PK₁ cells and ameliorated the increase in LDH leakage induced by CER. dBcAMP reduced the cell injury induced by CER. Our results suggest that a signalling pathway of cAMP protects against CER-induced renal cell injury, which is probably due to generation of oxygen radicals.

Keywords: cAMP and oxidative stress, Renal cell injury, Cephaloridine nephrotoxicity

Cephaloridine (CER) is a first-generation cephalosporin antibiotic that induces acute renal failure in humans and animals (1). The nephrotoxicity of CER is characterized by acute proximal tubular necrosis and is mainly dependent upon its accumulation and maintenance of higher concentration in the renal cortex (2). CER is taken up actively by the proximal tubular cells from blood via an organic anion transport system. However, the drug is able to leave the cells to a limited extent across the luminal membrane into the tubular fluid, resulting in a high intracellular concentration in the proximal tubule, which is nephrotoxic (3, 4). The biochemical mechanisms responsible for the proximal tubular necrosis in CER-induced nephrotoxicity have well been investigated and several lines of studies have indicated that CER undergoes a redox cycling reaction in renal microsomes to produce its intermediate. The intermediate forms oxygen radicals through oxidizing molecular oxygen. The reaction of the oxygen radicals with membrane lipids results in lipid peroxidation, leading to the development of CER nephrotoxicity (5).

Established renal epithelial cells have been recently used for toxicological research (6, 7). Williams et al. (8) have demonstrated that LLC-PK₁ cells have high sensitivity towards basolateral exposure to CER in terms of cellular viability assessed by nigrosin dye exclusion. In vitro model systems of ischemia-reperfusion injury of the kidney have also been reported using cultured renal epithelial cells, in which LLC-PK₁ was used as a model for renal proximal tubular epithelium cells. The LLC-PK₁ cells are more sensitive to hypoxic-reoxygenation injury than MDCK cells having characteristic distal tubular or cortical collecting duct epithelium (9).

Miyanoshita et al. (10) have demonstrated that a dibutyryl derivative of cAMP inhibits the production of oxygen radicals stimulated by phorbol 12-myristate 13-acetate, a protein kinase C activator, in rat glomeruli. Such a derivative of cAMP has also been reported to reduce the development of renal ischemia-reperfusion injury in which oxygen radicals have been suggested to be the potentially harmful factors (11). The purpose of this study was to evaluate the generation of lipid peroxidation in connection with CER-induced injury using LLC-PK₁ cells. We also tried to assess the protective effect of cAMP on such an injury.

LLC-PK₁ cells, a cultured renal epithelial cell line derived from porcine kidney (12), were maintained on plastic tissue-culture dishes containing Dulbecco's modified Eagle's medium and nutrient medium F-12 (1:1) (Gibco, Grand Island, NY, USA) supplemented with 5% fetal bovine serum (FBS). LLC-PK₁ cells were used between passages 127 and 136. A monolayer culture was grown in an atmosphere of 5% CO₂ – 95% air at 37°C. To obtain confluent cells, the cells were fed fresh medium without FBS on 4th day after being seeded. CER was added at a final concentration of 1 mM to the fresh medium. Indices of renal cell injury examined were lactate dehydrogenase (LDH) leakage from the cells into the media and lipid
peroxide level in the cells. At the end of incubation, the medium was collected for determination of LDH activity using a commercial kit (Wako Pure Chemical Industries, Ltd., Osaka). Cultured LLC-PK\textsubscript{1} cells were harvested by scraping them from the dish. The cells were suspended in medium containing 10 mM Tris-HCl buffer (pH 7.5) with 1 mM EDTA and then briefly sonicated before use. To evaluate the participation of oxygen radicals in the genesis of cell injury caused by CER, the level of lipid peroxidation in the cells was estimated by measuring the concentration of thiobarbituric acid-reactive substances (TBARS) as described by Buege and Aust (13). cAMP content was determined by a cyclic AMP kit YAMASA, which was purchased from Yamasa Corporation (Chyoushi). Protein content in LLC-PK\textsubscript{1} cells was determined by the method of Lowry et al. (14). The data are expressed as means ± S.E. Student’s t-test was used to evaluate the statistical significance of differences.

CER produced marked injury to LLC-PK\textsubscript{1} cells as reflected in the release of LDH into the medium at 36 hr (Fig. 1A). When the cells were exposed to the combination of CER and N,N\textsuperscript{-}dimethylthiourea (DMTU), a hydroxyl radical scavenger, the LDH release was substantially decreased compared to that due to exposure to CER alone, indicating that DMTU markedly suppressed CER-induced injury in LLC-PK\textsubscript{1} cells. DMTU alone did not affect LDH release. CER significantly increased the level of lipid peroxidation in LLC-PK\textsubscript{1} cells (Fig. 1B).

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**Fig. 1.** Effect of cephaloridine (CER) and N,N\textsuperscript{-}dimethylthiourea (DMTU) on lactate dehydrogenase (LDH) release into medium (A) and lipid peroxide (TBARS) level (B) in LLC-PK\textsubscript{1} cells. LLC-PK\textsubscript{1} cells were grown for 4 days and then given fresh serum-free medium with or without 1 mM CER and 30 mM DMTU. After 36 hr at 37°C, the cells and medium were collected separately for measurements of TBARS and LDH activity, respectively. Values each represent the mean ± S.E. of five to eight experiments. *P<0.05, **P<0.01, compared to the control value; #P<0.01, compared to CER alone.

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**Fig. 2.** Effect of cephaloridine (CER) and 3-isobutyl-1-methylxanthine (IBMX) on lactate dehydrogenase (LDH) release into medium (A) and lipid peroxide (TBARS) level (B) in LLC-PK\textsubscript{1} cells. LLC-PK\textsubscript{1} cells grown for 4 days were given fresh serum-free medium with or without 1 mM CER and 0.2 mM IBMX and then cultured for 36 hr at 37°C. Values each represent the mean ± S.E. of four experiments. *P<0.01, compared to the control value; †P<0.01, compared to CER alone.
Fig. 3. Changes in lactate dehydrogenase (LDH) release into the medium (A) and lipid peroxide (TBARS) level (B) in LLC-PK₁ cells exposed to 1 mM cephaloridine (CER) and 0.01 mM N⁶,²'-O-dibutyryl cAMP (dBcAMP) for 36 hr at 37°C. dBcAMP was added to the culture medium 2 hr before the addition of CER to the fresh serum-free medium. Values each represent the mean ± S.E. of four experiments. *P < 0.05 and **P < 0.01, compared to the control value; #P < 0.05 and ##P < 0.01, compared to CER alone.

DMTU abolished such an increase in lipid peroxidation, although DMTU alone had no effect on lipid peroxide level in cultured LLC-PK₁ cells.

Toxicity of CER to LLC-PK₁ cells as seen in the increased release of LDH activity was prevented by the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) (Fig. 2A). The CER-induced increase in lipid peroxidation was also lessened by IBMX (Fig. 2B). IBMX alone did not affect LDH release and lipid peroxidation. The cAMP content in the presence of CER for 24 hr in LLC-PK₁ cells was slightly less than the control value (5.54 ± 0.39 pmol/mg protein vs. 7.87 ± 0.36 pmol/mg protein, P < 0.005), but cAMP in the cells was increased not only by the coexistence of CER and IBMX (13.4 ± 0.55 pmol/mg protein) but also by IBMX alone (15.39 ± 0.82 pmol/mg protein).

In the next experiments, we examined the effect of N⁶,²'-O-dibutyryl cAMP (dBcAMP), a cAMP derivative capable of permeating the cell membrane, on the cell injury induced by CER. In a preliminary study, LLC-PK₁ cells were treated with different concentration (0.01, 0.025, 0.05, 0.5, 1 mM) of dBcAMP before the addition of CER. The lowest effective concentration of dBcAMP tested was 0.01 mM (data not shown), which was used in the next experiments. The leakage of LDH into the medium elevated by CER was significantly decreased by treatment of LLC-PK₁ cells with dBcAMP (Fig. 3A). The level of lipid peroxidation in the cells increased by CER was also depressed by dBcAMP (Fig. 3B). dBcAMP alone had no effect on either LDH release or lipid peroxidation.

The nephrotoxicity of CER has been shown to be mainly due to its accumulation in the kidney (3, 4). Previous data have demonstrated that CER generates oxygen radicals which react with membrane lipids, causing lipid peroxidation, thus contributing to the development of nephrotoxicity (5). The present experiment was designed to examine the participation of lipid peroxidation and cAMP in renal injury induced by CER using LLC-PK₁ cells. The results of this in vitro study clearly demonstrate that CER-induced injury is associated with the increase in lipid peroxidation in LLC-PK₁ cells. Such an increase in lipid peroxidation may play an important role in cell injury induced by CER in LLC-PK₁ cells. This in vitro finding using cultured cells is consistent with the involvement of lipid peroxidation in CER nephrotoxicity following administration to laboratory animals (5).

We also showed that IBMX and dBcAMP protected against cell injury induced by CER. Amelioration by IBMX and dBcAMP of the cell injury was accompanied by the decrease in lipid peroxidation in LLC-PK₁ cells exposed to CER. Our study using a cultured renal epithelial cell line suggests that cAMP in cells exerts a protective effect against renal cell injury caused by CER-induced oxidative stress. Koiwai et al. (11) have indicated in their in vivo experiments that dBcAMP protects against renal injury caused by ischemia-reperfusion. Aoyagi et al. (15) have also reported that dBcAMP and phosphodiesterase inhibitor depress the generation of oxygen radicals stimulated by puromycin aminonucleoside in isolated rat hepatocytes. dBcAMP has been found to inhibit oxygen radical production induced by phorbol 12-myristate 13-acetate in rat glomeruli (10). The present results together with those reported above suggest that cAMP protects against tissue injury by modifying the generation of oxygen radicals. Our in vitro models of nephrotoxicity using LLC-PK₁ cells may play a role in the elucidation of the mechanisms through which a signalling pathway of cAMP reduces the production of oxygen radicals.
REFERENCES