Hypoxia and Reoxygenation-Induced Injury of Renal Epithelial Cells: Effect of Free Radical Scavengers

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ABSTRACT—The aim of this study was to characterize injuries of LLC-PK1 and MDCK cells exposed to hypoxia and reoxygenation. Exposure of LLC-PK1 cells to hypoxia reduced the ATP contents and increased the leakage of lactate dehydrogenase (LDH), but MDCK cells had no such injuries. Hypoxia-reoxygenation of LLC-PK1 cells dramatically increased LDH leakage, which was suppressed by free radical scavengers, N,N'-diphenyl-p-phenylenediamine, superoxide dismutase and N,N'-dimethylthiourea. These results suggest that use of LLC-PK1 cells has advantages for the investigation of ischemia-reperfusion injury of the kidney as an in vitro model and that generation of oxygen radicals is involved in the cellular injury induced by hypoxia-reoxygenation.

Keywords: Oxidative stress, Hypoxia-reoxygenation, Renal epithelial cell

It is well known that ischemia followed by reperfusion of the kidney causes acute renal failure, as reflected by renal cell injury, obstruction of tubules and depression in glomerular filtration rate (1). Kotowski et al. (2) have reported that the second segments (S2) of proximal tubules of rat kidney have histologic changes induced by ischemia and reperfusion, suggesting selective damage of the proximal tubules. However, it is very difficult to understand the cellular mechanisms of such damage caused by kidney ischemia-reperfusion under in vivo conditions. Modern cell culture techniques enable renal epithelial cells to grow and differentiate into mature ones. Many established cell lines have been developed to display the feature of renal proximal tubular, distal tubular or collecting duct epithelium. LLC-PK1 and MDCK cells are derived from pig kidney and dog kidney, respectively, and have different features from each other. They have been recently used for physiological and toxicological research. Therefore, cultured renal cells would provide a malleable in vitro model to study cellular mechanisms of ischemia-reperfusion injury in the kidney.

Paller et al. (3) have demonstrated that lipid peroxidation mediated by oxygen radicals is involved in the development of the reperfusion injury in the ischemic kidney. Isolated renal tubules and primary cultures of renal cells have been shown to generate oxygen radicals under the conditions of hypoxia-reoxygenation (4, 5). In this report, we evaluated the use of two commercially available established lines of cultured renal epithelial cells subjected to hypoxia and reoxygenation as an in vitro model of ischemia-reperfusion injury in the kidney because of their easy handling. We also tried to reveal the protective effect of free radical scavengers on such an model.

LLC-PK1 cells, which were used between the 123rd to 131st passage, were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 5% fetal bovine serum (FBS) and 10 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES). MDCK cells at the 10th to 15th passage were grown in Eagle’s minimum essential medium (EMEM) containing 10% FBS and 10 mM HEPES. These cells appearing to grow in confluent monolayers were fed fresh medium on the 4th day after inoculation and exposed for 6 hr or 24 hr to the hypoxic condition using a Gas Pak Pouch™ (BBL Microbiology Systems, Cockeysville, MD, USA), in which the oxygen concentration was less than 2% within 2 hr after the exposure. In the case of reoxygenation, the cells were returned to a humidified atmosphere that was 95% air and 5% CO2 for 1 hr immediately after the hypoxic condition. After the exposure of the cells to hypoxia and reoxygenation, the medium was isolated and centrifuged at 3,000 rpm for 10 min at 5°C. The supernatants were used for the assay of lactate dehydrogenase (LDH) activity released from the cells, which was measured with a com-
mercial kit (Wako Pure Chemical Industries, Ltd., Osaka). The cells were washed twice and resuspended in 5% trichloroacetic acid. The cell suspension was homogenized for 15 sec and centrifuged at 3,500 rpm for 15 min at 5°C. The supernatants were used for the ATP assay, and the sediment was used for protein determination. The ATP level was measured by the luciferin-luciferase method of Kimmich et al. (6) with an ATP photometer (model 2000; JRB, Inc., La Jolla, CA, USA). The protein contents were assayed by the method of Bradford (7). N,N'-Diphenyl-p-phenylenediamine (DPPD), a strong antioxidant (8), was purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo), and it was dissolved in ethyl alcohol which was added to the medium to the final concentration of 1%. Copper-Zinc-containing superoxide dismutase (SOD) was provided by Asahi Chemical Industry Co., Ltd. The free radical scavengers, DPPD, SOD and N,N'-dimethylthiourea (DMTU), were added to the medium just before the beginning of hypoxia. The data are expressed as mean ± S.E. Student's t-test was used to evaluate the statistical significance of differences.

In the first experiment, we delineated how confluent monolayers of the two established cells, LLC-PK1 and MDCK, were injured by the exposure to the hypoxic condition as assessed by ATP contents and LDH release in the cells. The ATP contents were reduced time-dependently in LLC-PK1 cells exposed to the hypoxic condition (Fig. 1). The MDCK cells had the same ATP contents as did the LLC-PK1 cells. However, ATP contents in MDCK cells were not affected even after 24-hr hypoxia. The decrease in ATP contents caused by the hypoxic condition was returned to 12.78 ± 0.35 nmol/mg protein, which was about 80% of the 16.26 ± 0.42 nmol/mg protein of the control level, in LLC-PK1 cells exposed to hypoxia followed by 1-hr reoxygenation. Each value represents a mean ± S.E. of three experiments.

![Fig. 1](image1.png)

**Fig. 1.** Effect of hypoxia on ATP contents in LLC-PK1 and MDCK cells. LLC-PK1 cells and MDCK cells appearing in confluent monolayers on the 4th day after inoculation were exposed for 6 hr or 24 hr to a hypoxic atmosphere that had an oxygen concentration of less than 2%. The open column represents the ATP contents in the cells cultured under the normoxic atmosphere that had an oxygen concentration of less than 2%. The closed column represents those in the cells exposed to the hypoxic condition. Each value indicates a mean ± S.E. of three experiments. *P < 0.01, compared to the respective normoxic control.

![Fig. 2](image2.png)

**Fig. 2.** Effect of hypoxia and reoxygenation on LDH release from LLC-PK1 and MDCK cells. LLC-PK1 cells and MDCK cells were exposed to the hypoxic condition for 6 hr or to 6-hr hypoxia followed by 1-hr reoxygenation. Each value represents a mean ± S.E. of three experiments. *P < 0.01, compared to the respective normoxic control. #P < 0.01, compared to MDCK cells subjected to the same condition.

![Fig. 3](image3.png)

**Fig. 3.** LDH activity released from LLC-PK1 cells exposed to 7-hr normoxia (Control) or to 6-hr hypoxia followed by 1-hr reoxygenation with 10 μM N,N'-diphenyl-p-phenylenediamine (DPPD), 160 IU/ml superoxide dismutase (SOD) or 30 mM N,N'-dimethylthiourea (DMTU). Each drug was added to the culture medium just before hypoxia. Each value indicates a mean ± S.E. of three experiments. *P < 0.01, compared to the "normoxic" control. #P < 0.01, compared to the "None" control exposed to hypoxia followed by reoxygenation without free radical scavenger.
is adenylate cyclase, which is activated by parathyroid hormone in proximal tubular cells of intact kidney, is activated by both calcitonin and vasopressin but not by parathyroid hormone in LLC-PK₁ cells (14). So, these limitations of characteristics expressed by both cells in culture should be kept in mind when interpreting the results.

Paller et al. (3) have demonstrated the contribution of oxygen radicals to acute renal failure caused by ischemia-reperfusion in rats. In the present study, free radical scavengers, DMTU, SOD and DPPD, suppressed the cell injury induced by hypoxia followed by reoxygenation in LLC-PK₁ cells. The results suggest that oxidative stress is probably involved in one of the mechanisms of hypoxia-reperfusion injury in the cells. SOD may not diffuse into the cells so easily because of its molecular size. So, SOD may scavenge superoxide released from LLC-PK₁ cells on the surface of the cells. However, SOD may be able to scavenge superoxide inside cells, considering that isolated hepatocytes transport SOD into the cells by endocytosis (15). SOD catalyzes the conversion of superoxides, leading to the production of hydrogen peroxides, which are metabolized to water by glutathione peroxidase or catalase. An excess amount of hydrogen peroxides produced by hypoxia-reoxygenation in the cells may overwhelm the catalytic action of these enzymes and then could be converted into hydroxyl radicals that are scavenged by DMTU (9). We now intend to examine how superoxide production affects ATP levels to cause LDH leakage in LLC-PK₁ cells.

In conclusion, the use of the cultured renal epithelial cell line LLC-PK₁, which possesses the properties of renal proximal tubules, can be useful for investigating ischemia-reperfusion injury of the kidney as an in vitro model of such injury that is probably due to the generation of oxygen radicals.

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In vitro model systems of ischemia-reperfusion injury of the kidney have been demonstrated using isolated kidney, isolated renal tubules and primary cultures of renal cells. There has been, however, no attempt to apply established lines of cultured renal epithelial cells to the investigation of hypoxia-reoxygenation injury, although some studies on injury caused only by hypoxia have been reported using LLC-MK₂ and MDCK cells (10, 11). Here, the injurious effect of hypoxia on ATP contents in LLC-PK₁ cells was compared with those in MDCK cells. Interestingly, MDCK cells, which are characteristic of distal tubular or cortical collecting duct epithelium, were quite resistant to hypoxia even for 24 hr in terms of ATP loss, which has been shown to be a sensitive index of hypoxic stress (12). LLC-PK₁ cells were more markedly injured by hypoxia followed by reoxygenation than only by hypoxia as assessed by LDH leakage from the cells. Gower et al. (13) have described that the rapid depletion of ATP levels compromises calcium pumps to result in a gain of cellular calcium levels in tissues subjected to hypoxia-reoxygenation. And this is likely to lead to derangement of cell function. However, in this study, the depletion of ATP contents caused by 6-hr hypoxia did not increase LDH release from LLC-PK₁ cells to the medium; and 1-hr reoxygenation after hypoxia greatly enhanced LDH leakage from the cells, although the ATP contents in those cells restored to 80% of the control level. Further studies will be required to clarify whether ATP depletion in the cells exposed to hypoxia is important in accelerating LDH leakage from the cells exposed to hypoxia followed by reoxygenation.

In addition, LLC-PK₁ cells were more sensitive to hypoxia and reoxygenation than MDCK cells that were not injured by hypoxia and reoxygenation in this study. The high sensitivity of LLC-PK₁ cells, which are characteristic of proximal tubular epithelium, to hypoxia-reoxygenation is compatible with the sensitivity of proximal tubules to ischemia-reperfusion injury in whole kidney. These established cell lines do not express all of the characteristics of the derived epithelium of renal tubules, just as adenylate cyclase, which is activated by parathyroid hormone in proximal tubular cells of intact kidney, is activated by both calcitonin and vasopressin but not by parathyroid hormone in LLC-PK₁ cells (14). So, these limitations of characteristics expressed by both cells in culture should be kept in mind when interpreting the results.

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