Endonuclease-induced DNA Damage and Cell Death in Oxidant Injury to Renal Tubular Epithelial Cells

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Abstract

Hydrogen peroxide (H_2O_2)-induced DNA damage and cell death have been attributed to the direct cytotoxicity of H_2O_2 and other oxidant species generated from H_2O_2. We examined the possibility that oxidants activate endonucleases leading to DNA damage and cell death in renal tubular epithelial cells, similar to that described for apoptosis. Within minutes, H_2O_2 caused DNA strand breaks in a dose-dependent manner, followed by cell death. DNA fragmentation was demonstrated both by the release of [^{3}H]thymidine in 27,000-*g* supernatant as well as the occurrence of low molecular weight DNA fragments on agarose gel electrophoresis, characteristic of endonuclease cleavage. Endonuclease inhibitors, aurintricarboxylic acid, Evans blue, and zinc ion prevented H_2O_2-induced DNA strand breaks, fragmentation, and cell death. Inhibitors of protein or mRNA synthesis had only minor protection against H_2O_2-induced DNA damage in contrast to complete protection reported in apoptotic thymocytes. Micrococcal endonuclease induced similar DNA strand breaks in LLC-PK$_1$ cells, and the endonuclease inhibitors prevented the events confirming the ability of endonucleases to induce DNA damage. The protective effect of aurintricarboxylic acid was not due to the prevention of the rise in intracellular free calcium. We conclude that endonuclease activation occurs as an early event leading to DNA damage and cell death in renal tubular epithelial cells exposed to oxidant stress and, in contrast to apoptotic thymocytes, does not require macromolecular synthesis. (*J. Clin. Invest.* 1992. 90:2593–2597.) Key words: hydrogen peroxide • DNA damage • endonuclease • macromolecular synthesis • LLC-PK$_1$ cells

Introduction

Sequential reduction of oxygen along the univalent pathway leads to the generation of superoxide anion, hydrogen peroxide (H_2O_2), hydroxyl radical, and water (1, 2). These partially reduced oxygen intermediates have been implicated as important mediators of ischemic, toxic, and immune-mediated tissue injury (3–8). One of the earliest events after exposure of different cell types to oxidants is DNA strand breaks. H_2O_2 is the dominant oxidant leading to DNA strand breaks (9), presumably because it is both stable and able to freely penetrate the cell, thereby gaining access to DNA. However, H_2O_2 does not undergo any chemical reaction with DNA; hence, DNA damage cannot be the result of direct attack of H_2O_2 upon the DNA. It is generally accepted that the DNA damage results from the site-specific generation of hydroxyl radical (or a similar highly reactive oxidant) by the reaction of H_2O_2 with metal ions bound upon or very close to the DNA (9–13). In addition, in in vitro models of oxidant-induced cell death, hydroxyl radical scavengers and/or iron chelators (presumably because iron is critical in the generation of hydroxyl radicals via the metal-catalyzed Haber-Weiss reaction) have been shown to be protective (14–17). Thus, both the DNA damage and cell death have been attributed to the direct toxicity of the oxidants.

Programmed cell death or apoptosis is a process whereby cells die in a controlled manner, in response to specific stimuli, apparently following an intrinsic program (18–21). This process provides, for example, a mechanism for deletion of specific cell populations in the developing embryo. Much of our knowledge about the mechanisms underlying apoptosis comes from studies with lymphocytes or immature thymocytes, which readily undergo programmed cell death in response to glucocorticoid hormones (18–21). The process is characterized by several early morphological alterations, including plasma membrane blebbing and chromatin condensation (19). Endogenous endonuclease activation, resulting in the cleavage of host chromatin into oligonucleosome-length DNA fragments, is a characteristic biochemical marker for programmed cell death (18–20). Apoptosis requires that the dying cell be metabolically active, and apparently a rise in cytoplasmic calcium concentration appears to serve as a common early signal for the initiation of apoptosis (20, 21).

In our recent study using LLC-PK$_1$ cells (a renal tubular epithelial cell line) we demonstrated that in response to H_2O_2 there is an early rise in intracellular free calcium that precedes cell death and that intracellular calcium chelators prevent cell death (22). Based on this we considered the possibility that endonuclease activation may play an important role for DNA fragmentation and cell death in response to an oxidant stress. Thus, we examined the effect of H_2O_2 on DNA damage and cell death in LLC-PK$_1$ cells and whether the inhibition of endonucleases prevents these events. Our data demonstrate that endonuclease activation occurs as an early event leading to DNA damage and cell death in renal tubular epithelial cells exposed to oxidative stress.

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Methods

Incubation conditions. LLC-PK_1 cells (CRL 1392; American Type Culture Collection, Rockville, MD) were grown and maintained as previously described (17, 22) and after confluency were harvested with a rubber policeman for experiments.

Cells were washed and resuspended in serum-free media (DME) containing 1 mM Ca^{2+}, and then incubated with or without H_2O_2. At the end of incubation time indicated, catalase (800 U/ml; Boehringer Mannheim Biochemicals, Indianapolis, IN) was added to stop the reaction of H_2O_2. In some experiments, cells were preincubated with endonuclease inhibitors (20, 21, 23–26), aurintricarboxylic acid (10 μM), Evans blue (2 μg/ml), and zinc ion (50 μM), as well as fuchsin acid (50 μM), a structural analogue of aurintricarboxylic acid (24), for 30 min. In experiments using inhibitors of protein or mRNA synthesis, cells were preincubated with actinomycin D (5 μg/ml) or cycloheximide (10 μM) for 30 min. After the treatment cells were washed and resuspended in DME, then the desired concentration of H_2O_2 was added. In separate experiments with micrococcal endonuclease, cells resuspended in DME containing 1 mM Ca^{2+} were subjected to two cycles of freeze/thaw (dry ice/ethanol bath for 30 s followed by water bath at 37°C for 90 s), and incubated with micrococcal endonuclease (15 U/ml; Worthington Biochemical Corp., Freehold, NJ) for 30 min in the absence or presence of the endonuclease inhibitors.

Measurement of DNA double-strand breaks. The formation of single-strand DNA breaks from double-strand DNA was measured by the alkaline unwinding and ethidium bromide fluorescence as previously described (27). Ethidium bromide fluorescence was measured at 520 nm excitation and 590 nm emission using a fluorescence spectrophotometer (Perkin-Elmer Corp., Norwalk, CT). Under the conditions used, ethidium bromide binds preferentially to double-strand DNA. Percent double-strand DNA (D) was determined by the equation: $\%D = 100\times [F(P) - F(B)]/[F(T) - F(B)]$; where $F(P)$ is the sample fluorescence, $F(T)$ is the maximal fluorescence, and $F(B)$ is the background fluorescence.

DNA fragmentation assay. DNA fragmentation was determined by the method of Duke et al. (28), with some modification. LLC-PK_1 cells plated onto a six-well plate (Costar, Cambridge, MA) after confluency were incubated with 5 μCi per well of [^{3}H]thymidine (925 GBq/mmol; Amersham International, Amersham, UK) overnight at 37°C. Cells were washed with DME, incubated for 60 min on ice, and washed again before use. Then, the cells were incubated with 2 ml of serum-free media in the absence or presence of the relevant agents for the time indicated. At the end of the experiments, the incubation medium was carefully withdrawn and saved, and the cells were lysed with 2 ml of 25 mM sodium acetate buffer, pH 6.6. The lysates were centrifuged at 27,000 *g* for 20 min to separate intact chromatin (pellet) from fragmented DNA (supernatant). The radioactivities in the incubation medium, in the supernatant, and in the pellet were determined by a liquid scintillation counter (Packard Instrument Co., Inc., Meriden, CT). Specific DNA fragmentation was calculated by the formula: % specific DNA fragments = $100\times \text{cpm}_{\text{frags}}/\text{cpm}_{\text{total}}$; where $\text{cpm}_{\text{frags}}$ = the cpm in the incubation medium plus the cpm in the 27,000-*g* supernatant, and the $\text{cpm}_{\text{total}}$ = $\text{cpm}_{\text{frags}}$ plus the cpm in the 27,000-*g* pellet. The experimental data are subtracted from control value (no addition) at each time point.

Electrophoretic analysis of DNA fragmentation. Soluble DNA from LLC-PK_1 cells was extracted as previously described (29). After treatment of cells with the relevant agents, LLC-PK_1 cells were lysed in 0.5% Triton X-100, 0.5 mM Tris-HCl buffer (pH 7.4), and 20 mM EDTA for 30 min at 4°C. The lysates were centrifuged at 27,000 *g* for 20 min to separate the fragmented DNA (supernatant) from intact high molecular weight chromosomal DNA (pellet). The resulting supernatants were extracted with phenol-chloroform and precipitated with 0.3 M sodium acetate and 2.2 vol of ethanol. The nucleic acid from each supernatant was resuspended in 10 mM Tris-HCl and 1 mM EDTA, and the concentration was determined by UV absorbance at 260 nm. The same amount of nucleic acid from each sample (30 μg) was subjected to electrophoresis on a 2% agarose gel. The gel was incubated with RNase A (final concentration 20 μg/ml) at 37°C for 4 h before staining ethidium bromide.

Cell viability determination. Cell viability was determined by trypan blue exclusion (17, 22). Cells failing to exclude the dye were considered nonviable and the data are expressed as a percentage of the total.

Intracellular free calcium concentration measurement. Intracellular free Ca^{2+} concentration was measured by monitoring intracellular fura-2 fluorescence as in our previous study (22), but using a fluorescence spectrophotometer (F-2000; Hitachi Instrument Corp., Danbury, CT) with 340/380 nm excitation ratio and 500 nm emission (30). We confirmed that H_2O_2 by itself does not alter the Ca^{2+}-sensitive fura-2 signal.

Statistics. Results are means±SE. Statistical significance was determined by an analysis of variance with the Newman-Kleus procedure.

Results

Hydrogen peroxide caused DNA double-strand breaks in LLC-PK_1 cells in a dose-dependent manner (0.1–5 mM; Fig. 1 *A*) within minutes after exposure to H_2O_2 (Fig. 1 *B*). Based on

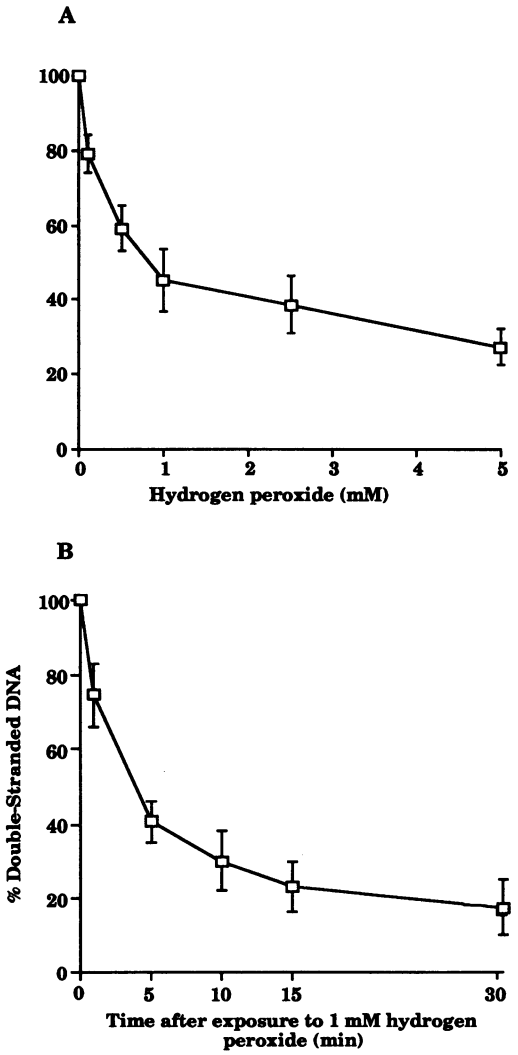

Figure 1. (*A*) Concentration-dependent effect of H_2O_2 on DNA double-strand breaks (at 10 min after exposure) in LLC-PK_1 cells. Results are means±SE (n = 3). (*B*) Time course of effect of hydrogen peroxide (H_2O_2, 1 mM) on DNA double-strand breaks in LLC-PK_1 cells as measured by alkaline unwinding and ethidium bromide fluorescence assay. Results are means±SE (n = 3).

these data, 1 mM H_2O_2 was utilized in the studies and the DNA double-strand breaks were determined at 10 min after exposure to H_2O_2 (except where otherwise stated).

Aurintricarboxylic acid, Evans blue, and zinc ion have been shown to inhibit endogenous endonuclease activity and utilized to delineate a role for endonucleases in DNA fragmentation (20, 21, 23–26). To evaluate a role of endonucleases in H_2O_2-induced DNA damage, we examined the effect of the endonuclease inhibitors on H_2O_2-induced DNA damage in LLC-PK_1 cells. The three different endonuclease inhibitors, aurintricarboxylic acid, Evans blue, and zinc ion, prevented H_2O_2-induced DNA double-strand breaks in LLC-PK_1 cells, whereas fuchsin acid, a structural analogue of aurintricarboxylic acid (24), had no effect (Fig. 2 *A*). We confirmed that the endonuclease inhibitors did not scavenge H_2O_2 (data not shown; 31).

Ca^{2+}-dependent micrococcal endonuclease (15 U/ml) produced similar DNA double-strand breaks in permeabilized LLC-PK_1 cells by freeze/thaw from 86±1% (no addition) to 50±4%, which was prevented by the endonuclease inhibitors, 10 μM aurintricarboxylic acid (79±3%, $P < 0.01$), 2 μg/ml Evans blue (66±1%, $P < 0.01$), and 50 μM $ZnSO_4$ (88±1%, $P < 0.01$), but not by 50 μM fuchsin acid (45±6%, $n = 3$), confirming the ability of endonucleases to induce DNA strand breaks in LLC-PK_1 cells.

Apoptosis in thymocytes has been suggested to be dependent on ongoing macromolecular synthesis because inhibitors of protein or mRNA synthesis block DNA fragmentation in thymocytes exposed to glucocorticoids (19, 20). However, inhibitors of protein or mRNA synthesis, actinomycin D and cycloheximide, had only minor protection against H_2O_2-induced DNA double-strand breaks in LLC-PK_1 cells (Fig. 2 *B*), similar to that reported in other tissues in response to mild hyperthermia (32), but in contrast to almost complete protection reported in apoptotic thymocytes (19–21).

In apoptosis or programmed cell death endonuclease activation cleaves host DNA into oligonucleosome-length fragments (18–20). We examined whether H_2O_2 produces endonuclease-induced DNA fragmentation in LLC-PK_1 cells. While no DNA fragmentation was detected as determined by release of [^{3}H]thymidine in 27,000-*g* supernatant at 2 h after exposure to 1 mM H_2O_2, it was detectable at 3 h and increased thereafter (Fig. 3 *A*). In separate studies we demonstrated that H_2O_2 caused the DNA fragmentation into multiples of low molecular weight DNA (~ 200 bp) (Fig. 3 *B*). Previous studies have shown that such fragmentation is characteristic of endonuclease cleavage of DNA observed in apoptotic thymocytes or lymphocytes (18–20) and is not an artifact of the process of lysis (18, 19). The endonuclease inhibitors prevented the DNA fragmentation due to H_2O_2 (Fig. 3 *B*), suggesting a role for endonucleases in H_2O_2-induced DNA fragmentation.

In separate experiments, we examined the effect of inhibition of endonucleases on H_2O_2-induced cell death as measured by trypan blue exclusion. On the basis of our previous study (22), 60, 90, and 120 min after exposure to 1 mM H_2O_2 were chosen as time points to be examined. The endonuclease inhibitors, aurintricarboxylic acid (10 μM), Evans blue (2 μg/ml), and $ZnSO_4$ (50 μM), prevented the cell death due to H_2O_2, whereas fuchsin acid (50 μM) had no effect (Fig. 4). We confirmed that the endonuclease inhibitors used did not interfere with the trypan blue exclusion assay (data not shown).

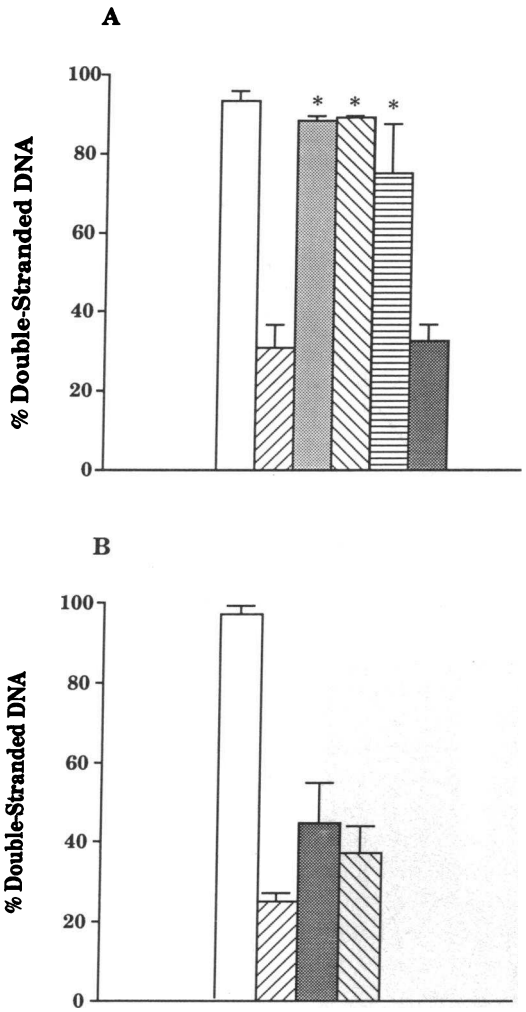

Figure 2. (*A*) Effect of endonuclease inhibitors on H_2O_2-induced DNA double-strand breaks in LLC-PK_1 cells. Cells were incubated with the endonuclease inhibitors for 30 min, washed, and resuspended in DME, and then exposed to 1 mM H_2O_2 for 10 min. Shown are control (no addition) (□), 1 mM H_2O_2 (▨), 10 μM aurintricarboxylic acid (▨), 2 μg/ml Evans blue (■), 50 μM $ZnSO_4$ (▩), and 50 μM fuchsin acid (■). Results are means±SE ($n = 3$). *$P < 0.01$, compared with H_2O_2 only. (*B*) Effect of protein or mRNA synthesis inhibitors on H_2O_2-induced DNA double-strand breaks in LLC-PK_1 cells. Cells were pretreated with inhibitors of protein or mRNA synthesis, actinomycin D or cycloheximide, for 30 min, washed, and exposed to H_2O_2 for 10 min. Shown are control (no addition) (□), 1 mM H_2O_2 (▨), 5 μg/ml actinomycin D (■), and 10 μM cycloheximide (■). Results are means±SE ($n = 3$).

We also examined the effect of a lower dose of H_2O_2 (0.5 mM) with a longer incubation period on DNA fragmentation and cell death. After pretreatment with or without an endonuclease inhibitor, aurintricarboxylic acid (10 μM), for 30 min, LLC-PK_1 cells were washed and exposed to 0.5 mM H_2O_2 for 8 h, and then DNA fragmentation (as measured by [^{3}H]thymidine release) and cell death (trypan blue exclusion) were examined. Hydrogen peroxide (0.5 mM)–induced DNA fragmentation (15±2%, $n = 2$), was almost completely prevented by aurintricarboxylic acid (1±1%, $n = 2$). Aurintricarboxylic acid was also protective against the cell death as measured by trypan blue exclusion (no addition, 9±2%; 0.5 mM

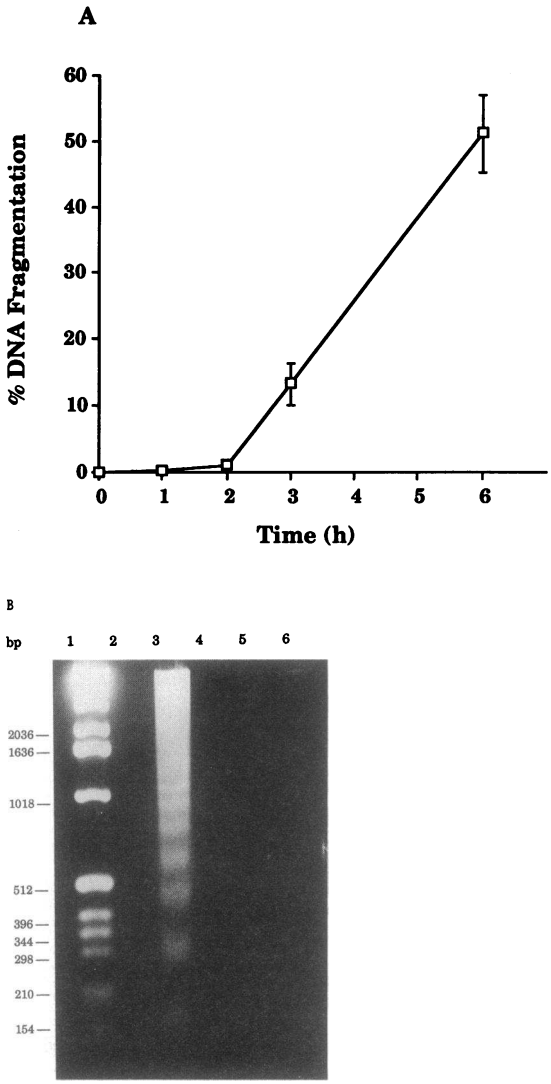

Figure 3. (*A*) Time course of effect of H_2O_2 on DNA fragmentation in LLC-PK_1 cells. DNA fragmentation was determined at various time points after exposure to 1 mM H_2O_2 as detailed in the text. Percent specific DNA fragments = 100× cpm_{frags}/cpm_{total}; where cpm_{frags} = the cpm in the incubation medium plus the cpm in the 27,000-*g* supernatant, and the cpm_{total} = cpm_{frags} plus the cpm in the 27,000-*g* pellet. The experimental data are means±SE (n = 4–6). (*B*) DNA fragmentation in LLC-PK1 cells 8 h after exposure to 1 mM H_2O_2 with or without endonuclease inhibitors. Fragmented DNA was obtained by centrifugation and precipitation as detailed in the text. The nucleic acid from each sample (30 μg) was subjected to electrophoresis on a 2% agarose gel. The gel was incubated with RNase A (20 μg/ml) at 37°C for 4 h before staining ethidium bromide. Lane *1*, ladder; lane *2*, control (no addition); lane *3*, H_2O_2 only; lane *4*, aurintricarboxylic acid (10 μM)–treated sample; lane *5*, Evans blue (2 μg/ml)–treated sample; lane *6*, $ZnSO_4$ (50 μM)-treated sample.

H_2O_2, 35±1%; 0.5 mM H_2O_2 plus aurintricarboxylic acid, 11±2%, n = 2).

We have previously shown that in LLC-PK_1 cells H_2O_2 caused a sustained rise in intracellular free calcium and that chelating intracellular calcium prevented cell death (22). After a 60-min exposure, H_2O_2 (1 mM) induced a similar rise in intracellular free calcium in the presence (756±50 nM) or absence (626±12 nM) of 10 μM aurintricarboxylic acid with control (no addition) being 140±19 nM (n = 3). These data indicate that the protective effect of aurintricarboxylic acid on H_2O_2-induced DNA damage and cell death is not due to the prevention of the rise in intracellular free calcium.

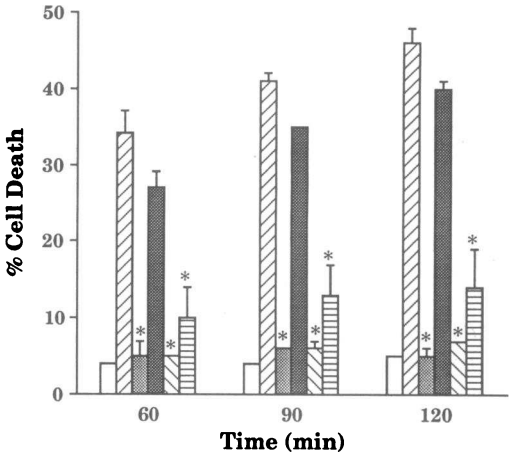

Figure 4. Effect of endonuclease inhibitors on H_2O_2-induced cell death as measured by trypan blue exclusion in LLC-PK_1 cells. Cells were preincubated with the endonuclease inhibitors for 30 min, then washed and resuspended in DME containing 1 mM Ca^{2+}, and incubated with H_2O_2. At the end of incubation, catalase (800 U/ml) was added to stop the reaction of H_2O_2. The endonuclease inhibitors did not affect the cell viability or trypan blue exclusion assay. Results are means±SE and nonviable cells are expressed as a percentage of the total. Shown are control (no addition) (□), 1 mM H_2O_2 (▨), 10 μM aurintricarboxylic acid (□), 50 μM fuchsin acid (■), 2 μg/ml Evans blue (▧), and 50 μM $ZnSO_4$ (▤) (n = 3). *$P < 0.01$, compared with H_2O_2 only.

Discussion

In the present study, we have shown that in LLC-PK_1 cells H_2O_2 caused significant DNA double-strand breaks that preceded cell death. In addition, exposure of LLC-PK_1 cells to H_2O_2 led to DNA fragmentation characteristic of endonuclease-induced DNA damage as seen in apoptotic thymocytes or lymphocytes (18–20). Previous studies have shown that such fragmentation is characteristic of endonuclease cleavage of DNA and is not an artifact of the process of lysis (18, 19). The inhibition of endonuclease activity using aurintricarboxylic acid, Evans blue, and zinc ion prevented H_2O_2-induced DNA strand breaks, fragmentation, and cell death. The protective effect of an endonuclease inhibitor, aurintricarboxylic acid, on H_2O_2-induced DNA damage and cell death was not due to the prevention of the rise in intracellular free calcium due to H_2O_2. In addition, the endonuclease inhibitors did not scavenge H_2O_2 and did not interfere with the trypan blue exclusion assay (data not shown). Taken together, the data indicate that H_2O_2 results in endonuclease activation leading to DNA fragmentation and cell death in LLC-PK_1 cells. This is further supported by the findings that a Ca^{2+}-dependent micrococcal endonuclease produces similar DNA strand breaks in permeabilized LLC-PK_1 cells by freeze/thaw. Our findings may be relevant to those models of renal injury where reactive oxygen metabolites have been postulated to be important. Indeed, in a recent study it was reported that there was morphological and biochemical

evidence of apoptosis during reperfusion after renal ischemia (33).

Apoptotic cell death has been shown to be dependent on ongoing protein or mRNA synthesis because inhibitors of protein or mRNA synthesis almost completely block DNA fragmentation and cell death in thymocytes exposed to glucocorticoids (19, 20). In contrast to this, an inhibition of protein or mRNA synthesis using actinomycin D or cycloheximide provided only minor protection against H_2O_2-induced DNA damage in LLC-PK_1 cells. Thus, it appears that, as in some other tissues (32), H_2O_2-induced endonuclease activation in LLC-PK_1 cells does not require macromolecular synthesis. These data also suggest that the regulation of endogenous endonucleases is dependent on the nature of the stimuli and/or the cell type.

In summary, our data demonstrate that endonuclease activation occurs as an early event leading to DNA damage and cell death in renal tubular epithelial cells exposed to oxidative stress and, in contrast to apoptotic thymocytes, does not require macromolecular synthesis.

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