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Endonuclease induced DNA damage and cell death in chemical hypoxic injury to LLC-PK_1 cells

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Endonuclease induced DNA damage and cell death in chemical hypoxic injury to LLC-PK_1 cells. Hypoxia is considered to result in a necrotic form of cell injury. We examined the role of endonuclease activation, considered a feature of apoptosis, in DNA damage and cell death in hypoxic injury in LLC-PK_1 cells. Hypoxia in LLC-PK_1 cells was induced using a combination of glucose deprivation and a mitochondrial inhibitor, antimycin A (10 μM). Chemical hypoxia caused DNA damage as measured by the alkaline unwinding assay and internucleosomal DNA fragmentation that preceded cell death. Incubating protein extract of cells subjected to chemical hypoxia with calf thymus DNA resulted in oligonucleosome length fragments, which were prevented by an endonuclease inhibitor, aurintricarboxylic acid. Chemical hypoxia resulted in an increased DNA degrading activity with a molecular mass of aproximately 15 kDa. Endonuclease inhibitors, aurintricarboxylic acid and Evans blue, prevented antimycin A-induced DNA strand breaks, fragmentation and cell death. We conclude that endonuclease activation plays an important role in chemical hypoxic injury to LLC-PK_1 cells.

Ischemia/reperfusion *in vivo* and hypoxia/reoxygenation *in vitro* are classically considered to result in a necrotic form of cell death in which there is rapid collapse of internal homeostasis of the cell [1–3] associated with membrane lysis and inflammation [4]. In contrast, apoptosis, or programmed cell death, is considered operationally, morphologically and biochemically distinct from necrotic cell death [1, 5–7], and is a process in which the cell actively participates in its own demise. Endogenous endonuclease activation, resulting in the cleavage of host chromatin into oligonucleosome-length DNA fragments, has been considered a characteristic biochemical marker for apoptosis [4, 8]. We have recently shown that subjecting rat renal proximal tubules to hypoxia/reoxygenation results in DNA strand breaks and DNA fragmentation which precedes cell death [9]. We demonstrated that endonuclease activation is an early event which is entirely responsible for the DNA damage and partially responsible for the cell death that occurs during hypoxia/reoxygenation injury. Despite unequivocal evidence of endonuclease activation, the morphologic features of apoptosis including chromatin condensation were not observed by light and electron microscopy, indicating that endonuclease activation does not necessarily induce chromatin condensation, and that DNA fragmentation and chromatin condensation are likely to be triggered through separate pathways during apoptosis.

Cultured cells are very valuable for accessing mechanistic issues and have been extensively utilized to study hypoxic injury [2, 10, 11]. Because of the difficulties in incurring hypoxic injury to cultured cells, chemical hypoxia which involves the use of metabolic inhibitors to induce a severe ATP depletion characteristic of ischemia and other oxygen deprivation states has been utilized [2, 10]. The role of endonuclease activation in chemical hypoxia-induced injury in renal tubular epithelial cells has not been previously examined. In the present study, we examined the role of endonuclease activation in DNA damage and/or cell death in LLC-PK_1 cells subjected to chemical hypoxia.

Methods

Cell culture

LLC-PK_1 cells, renal tubular epithelial cells, obtained from the American Type Culture Collection (Rockville, MD, USA) were cultured as in our previous studies [12]. They were grown in 75 cm^2 plastic culture flasks containing Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 20 mM 4-(2-hydroxy ethyl)-1-piperazine ethanesulfonic acid (HEPES) and 2 mM non essential amino acids. Cultures were maintained in a humidified incubator gassed with 5% CO_2–95% air at 37°C and fed at intervals of 48 to 72 hours. Experiments were performed two to three days post-confluency.

Determination of DNA damage

The residual double strand DNA was measured by the alkaline unwinding assay and determination of ethidium bromide fluorescence according to the method of Birnboim and Jevcak [13] as utilized in our previous studies [9, 12]. Ethidium bromide fluorescence was measured at 520 nm excitation and 590 nm emission using a fluorescence spectrophotometer (PTI, Photon Technology International Co., South Brunswick, NJ, USA). Under the conditions employed, ethidium bromide binds preferentially to double strand DNA. % 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 total fluorescence prior to alkaline treatment, and F(B) is the background fluorescence that was obtained from sonicated, alkaline-treated DNA, a condition under which the DNA is completely unwound and represents the

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fluorescence due to all components other than double stranded DNA.

Detection of DNA fragmentation by agarose gel electrophoresis

Fragmented DNA was isolated from LLC-PK_1 cells as previously described [8, 12, 14]. Cells were harvested by centrifugation and the pellets were lysed in 0.5% Triton X-100, 10 mM Tris-HCl, pH 8.0, 25 mM EDTA for one hour at 4°C. The lysates were centrifuged at 16,000 g for 20 minutes to separate the fragmented DNA (supernatant) from the intact DNA (pellet). The resulting supernatant containing fragmented DNA was incubated with proteinase K (200 μg/ml) at 50°C overnight and then extracted with phenol-chloroform-isoamyl alcohol (50:49:1) mixture. The fragmented DNA in the aqueous phase was precipitated with 0.1 vol of 3 M sodium acetate and 2.5 vol of 100% ethanol at −20°C. The DNA was dissolved in 10 mM Tris-HCl and 0.1 mM EDTA. The concentration of nucleic acid in each sample was determined by UV absorbance at 260 nm. The same amount of nucleic acid from each sample was subjected to electrophoresis on 1.6% agarose gel. After electrophoresis the gel was incubated with RNase (20 μg/ml) at 37°C for three hours. DNA was visualized by UV fluorescence after staining with ethidium bromide (0.5 μg/ml).

Endonuclease activity assay

Endonuclease activity was detected by SDS-polyacrylamide gel electrophoresis as previously described [9, 15, 16]. LLC-PK_1 cells were pelleted by centrifugation and suspended in 100 mM Tris-HCl, pH 7.6 and homogenized at 4°C followed by sonication. Crude protein extract was obtained by centrifugation of the lysate at 16,000 g for five minutes. Protein concentration was determined by the method of Bio-Rad protein assay [17]. Protein aliquot (20 μg) from each sample was mixed with sample loading buffer (7.5% glycerol, 1% SDS, 50 mM Tris-HCl, pH 6.8, 1% mercaptoethanol, 5 mM dithiothreitol) before loading into the gel. Protein samples are electrophoresed using a vertical slab gel consisting of 4% stacking gel, 15% separating gel and heat denatured calf thymus DNA (10 μg/ml). Micrococcal nuclease (0.2 ng/lane, Worthington, NJ, USA) was used as a positive control. After electrophoresis, the gel was washed overnight in washing buffer (40 mM Tris-HCl, pH 7.6, 2 mM $MgCl_2$), then the gel was incubated for two to three hours in fresh washing buffer in the presence of 2 mM $CaCl_2$ and stained with ethidium bromide (1 μg/ml).

Determination of cell injury

Cell viability was determined using trypan blue exclusion [12] and LDH release [18] as previously described.

Experimental protocols

Hypoxia was induced using a combination of glucose deprivation and mitochondrial electron transport inhibition as previously described [2, 10]. After confluency, LLC-PK_1 cells were rinsed with DMEM without serum, glutamine, pyruvate, non-essential amino acids or glucose, containing 3.7 g/liter $NaHCO_3$ and 20 mM HEPES at pH 7.4. Then the cells were incubated in the same glucose-free DMEM either with or without antimycin A for the period of time indicated. In initial studies we determined the optimum exposure time and the suitable concentration of antimycin A. To delineate the role of endonuclease, several endonuclease inhibitors, aurintricarboxylic acid (10 μM), Evans blue (2 μg/ml), and zinc sulfate (50 μM) were used. Fuchsin acid (50 μM), a structural analog of aurintricarboxylic acid, with little endonuclease inhibitory activity was used as a control. For the alkaline unwinding assay experiments, cells were preincubated with endonuclease inhibitors for 30 minutes, washed and exposed to antimycin A (10 μM) for 60 minutes. For cell injury experiments, cells were exposed to antimycin A in the presence of aurintricarboxylic acid or Evans blue for 120 to 180 minutes.

We considered the possibility that the effect of the endonuclease inhibitors may be due to the effect on antimycin A uptake. We, therefore, determined if endonuclease inhibitors affected reduction in ATP level produced by antimycin A. Cellular ATP was measured by luciferin/luciferase assay as previously described [19].

Materials

Aurintricarboxylic acid and Evans blue were from Aldrich Chemical Co. (Milwaukee, WI, USA). Acrylamide and bisacrylamide were obtained from Bio-Rad Laboratories (Hercules, CA, USA) and Eastman Kodak Co. (Rochester, NY, USA). Glucose-free DMEM was purchased from Gibco Life Technologies, Inc. (Gaithersburg, MD, USA). All other reagents were obtained from Sigma Chemical Co. (St. Louis, MO, USA), and were of the highest commercial grade available.

Statistics

Results are means ± SE. Statistical significance was determined by the Student's *t*-test. $P < 0.05$ was considered statistically different.

Results

Antimycin A-induced DNA strand breaks and cell death in LLC-PK_1 cells

As shown in Figure 1A, antimycin A (10 μM) caused significant DNA strand breaks in LLC-PK_1 cells in a time-dependent manner. Significant strand breaks occurred at 30 minutes after exposure of LLC-PK_1 cells and progressively increased up to 120 minutes where residual double stranded DNA was 39 ± 7% compared to 87 ± 5% in control cells, ($N = 4$, $P < 0.05$). The effect of antimycin A was dose-dependent (0.1 to 10 μM; Fig. 1B) with 44 ± 2% ($N = 6$, $P < 0.0002$) residual double stranded DNA obtained at 60 minutes with a concentration of 10 μM antimycin A, the highest concentration examined.

Based on these experiments, we examined the effect of 10 μM antimycin A on cell death as measured by trypan blue exclusion and LDH release. As shown in Figure 1C, antimycin A caused a marked and a significant increase in cell death as measured by trypan blue exclusion beginning 120 minutes after exposure (control 7 ± 1%; antimycin A 19 ± 1%; $N = 4$, $P < 0.001$) and increasing up to 180 minutes (control 9 ± 2%; antimycin A 24 ± 2%; $N = 4$ to 7, $P < 0.001$). Similar data were obtained using LDH release as a measure of cell death (Fig. 1D).

Role of endonuclease activation in antimycin A induced DNA damage

To obtain evidence for a potential role of endonuclease in the DNA damage, we sought evidence of DNA fragmentation by utilizing agarose gel electrophoresis. Exposure of LLC-PK_1 cells

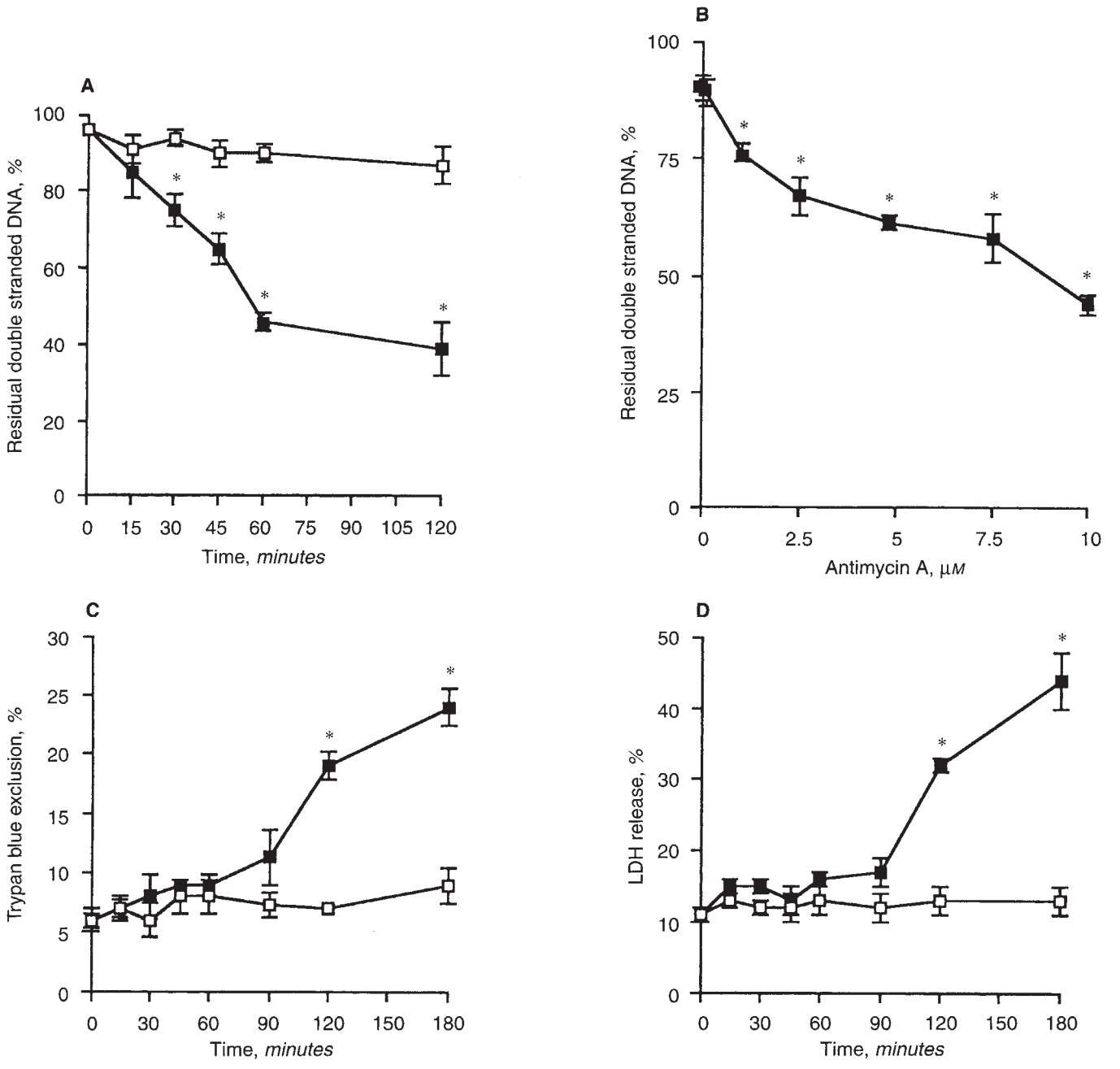

Fig. 1. A. *Time course of effect of antimycin A on DNA strand breaks in LLC-PK$_1$ cells.* Cells were exposed to antimycin A (10 μM) for various periods of time (0 to 2 hr). Results are means ± SE, $N = 4$ to 7. *$P < 0.05$, compared to control. **B.** Dose-dependent effect of antimycin A on DNA strand breaks in LLC-PK$_1$ cells. Cells were exposed to various doses of antimycin-A (0.1 to 10 μM) for 60 minutes. Results are means ± SE, $N = 5$. *$P < 0.002$, compared to control cells. **C.** Time course of effect of antimycin A on cytotoxicity as determined by trypan blue exclusion in LLC-PK$_1$ cells. Cells were exposed to antimycin A (10 μM) for various periods of time (0 to 3 hr). Results are means ± SE, $N = 4$ to 7. *$P < 0.001$, compared to control. **D.** Time course of effect of antimycin A on cytotoxicity as determined by LDH release in LLC-PK$_1$ cells. Results are means ± SE, $N = 3$ to 7. *$P < 0.0001$, compared to control. Shown are control (□) and antimycin A-treated cells (■).

to 10 μM antimycin A resulted in multiples of low molecular weight DNA fragments (~200 bp; Fig. 2). The DNA fragmentation was observed as early as 30 minutes (lane 2) and increased up to four hours (lane 5) after exposure to antimycin A and clearly preceded cell death. In contrast, after treatment of LLC-PK$_1$ cells with 0.005% Triton X-100 in serum-free DMEM for 60 minutes, there was no DNA fragmentation despite significant cell death as measured by trypan blue exclusion (control 13 ± 1%; Triton-treated 36 ± 3%, $N = 3$, $P < 0.0004$), indicating that DNA fragmentation was not a nonspecific effect resulting from cell death.

Aurintricarboxylic acid and Evans blue have been shown to inhibit endogenous endonuclease activity and have been utilized to delineate a role for endonucleases in DNA damage [8, 12, 20].

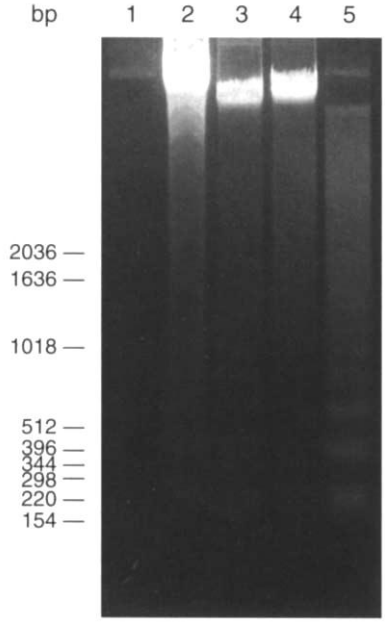

Fig. 2. *Time course of effect of antimycin A on DNA fragmentation in LLC-PK$_1$ cells.* Cells were exposed to antimycin A (10 μM) for different time points. The same amount of nucleic acid isolated from each sample was subjected to electrophoresis on 1.6% agarose gel. (1) Control; (2) 30 minutes; (3) one hour; (4) two hours; and (5) four hours.

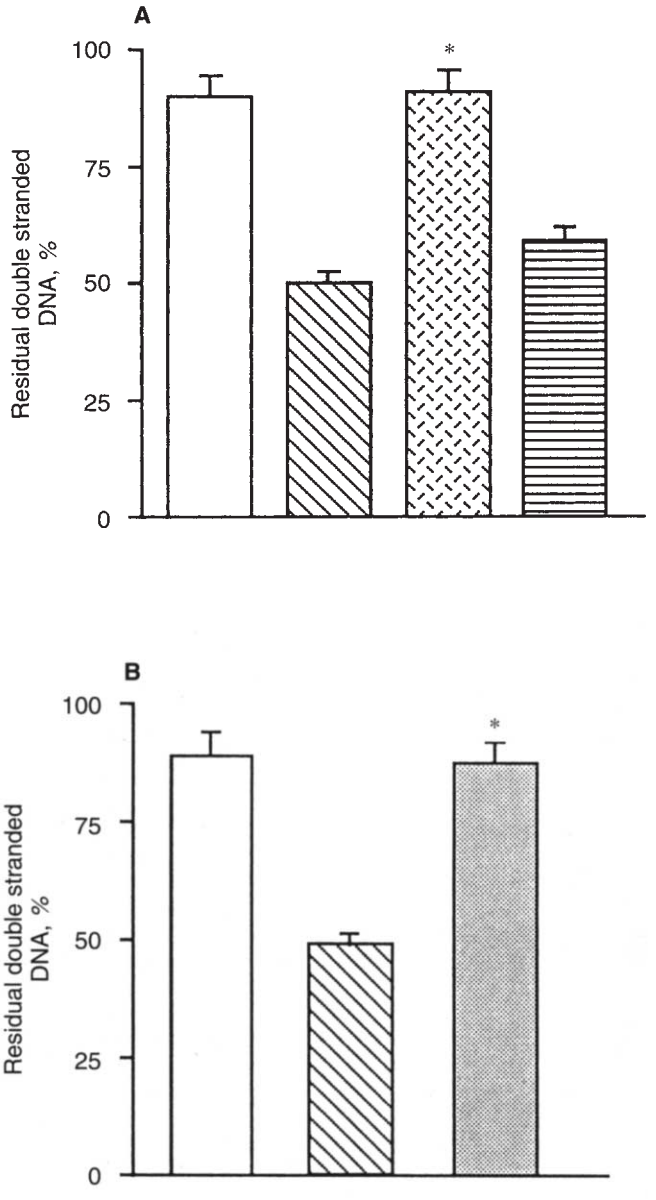

Fig. 3. *Effect of endonuclease inhibitors on antimycin A-induced DNA strand breaks in LLC-PK$_1$ cells.* **A.** Cells were preincubated with aurintricarboxylic acid (10 μM) or fuchsin acid (50 μM), a structural analog of aurintricarboxylic acid for 30 minutes, washed and then exposed to antimycin A (10 μM) for 60 minutes. Results are means ± SE, $N = 4$ to 6. *$P < 0.0001$, compared to cells exposed to antimycin A alone. Shown are control (□), antimycin A alone (▨), antimycin A plus aurintricarboxylic acid (▩), and antimycin A plus fuchsin acid (▤). **B.** Cells were preincubated with Evans blue (2 μg/ml) for 30 minutes, washed, and then exposed to antimycin A (10 μM) for 60 minutes. Results are means ± SE, $N = 6$. *$P < 0.0001$, compared to cells exposed to antimycin A alone. Shown are control (□), antimycin A alone (▨), and antimycin A plus Evans blue (▤).

Aurintricarboxylic acid provided almost complete protection against antimycin A-induced DNA strand breaks (residual double stranded DNA, 91 ± 3%, compared to cells treated with antimycin A alone, 50 ± 2%; $N = 6$, $P < 0.0001$; Fig. 3A). In contrast, fuchsin acid, a structural analog of aurintricarboxylic acid, with little endonuclease inhibitory activity [21], did not have any protective effect. Evans blue also provided complete protection against antimycin A-induced DNA strand breaks (residual double stranded DNA 87 ± 3%) compared to cells treated with antimycin A alone (47 ± 5%; $N = 6$, $P < 0.0001$; Fig. 3B). We next examined the effect of endonuclease inhibitors on antimycin A-induced DNA fragmentation. As shown in Figure 4, aurintricarboxylic acid, Evans blue and zinc prevented antimycin A-induced DNA fragmentation. It is conceivable that the effect of the endonuclease inhibitors could be due to a blockade in antimycin A uptake. If this was the case, then the fall in ATP level induced by antimycin A would be reduced in the presence of endonuclease inhibitors. There was a similar and dramatic fall in ATP levels in the presence of endonuclease inhibitors (data not shown), suggesting that the effect of endonuclease inhibitors was not due to the effect on antimycin A uptake. These data provide further evidence for the role of endonuclease(s) in antimycin A-induced DNA damage.

We examined the ability of the protein extract of antimycin A-treated cells to cleave calf thymus DNA into small fragments. As shown in Figure 5 incubation of the protein extract of antimycin A-treated cells with calf thymus DNA led to a time-dependent DNA fragmentation. Furthermore, an endonuclease inhibitor, aurintricarboxylic acid, was able to prevent the DNA fragmentation (data not shown). We also examined changes in DNA-degrading activity using SDS-polyacrylamide substrate gel with denatured DNA as the substrate. Protein extracts from LLC-PK$_1$ cells had DNA degrading activity with a molecular mass of approximately 15 kDa. This degrading activity was significantly increased in protein extracts from cells subjected to antimycin A as compared to control (Fig. 6).

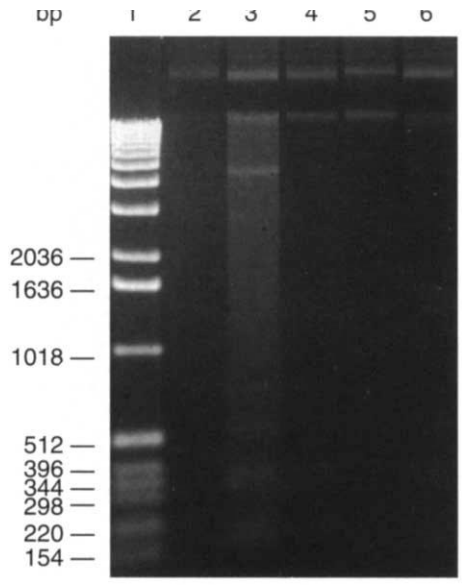

Fig. 4. *Effect of endonuclease inhibitors on antimycin A-induced DNA fragmentation in LLC-PK$_1$ cells.* Cells were exposed to antimycin A (10 μM) for 120 minutes. in the absence or presence of aurintricarboxylic acid (10 μM), Evans blue (2 μg/ml), or zinc sulfate (50 μM). (1) Molecular size marker, (2) control, (3) antimycin A alone, (4) antimycin A plus aurintricarboxylic acid, (5) antimycin A plus Evans blue, and (6) antimycin A plus zinc.

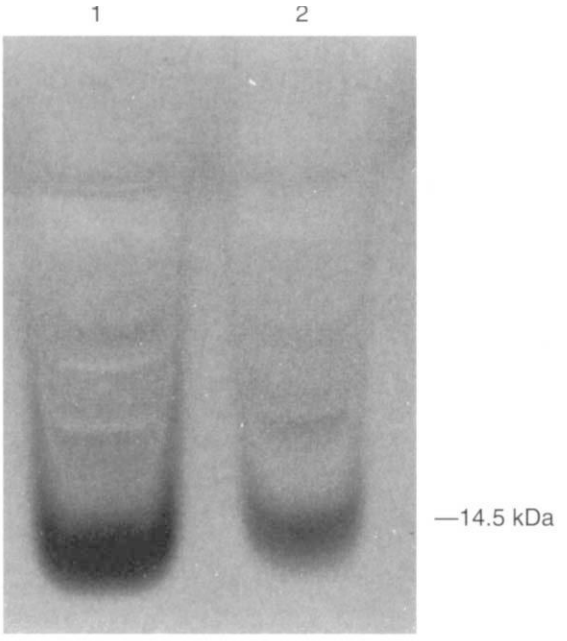

Fig. 6. *Effect of antimycin A on endonuclease activity in LLC-PK$_1$ cells.* Cells were exposed to antimycin A (10 μM) for 60 minutes. The same amount of protein extract (20 μg/lane) from each sample was applied to SDS-polyacrylamide gel containing 10 μg/ml heat-denatured calf thymus DNA. (1) Antimycin A treated cells and (2) control.

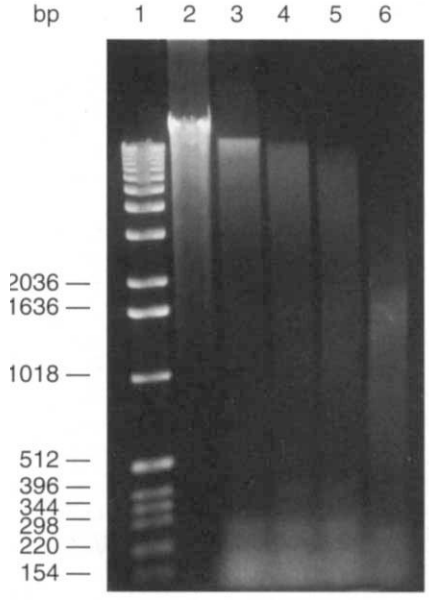

Fig. 5. *Effect of protein extract of antimycin A treated cells on calf thymus DNA.* The protein extract (10 μg) obtained from antimycin A treated cells for 90 minutes was incubated with 5 μg calf thymus DNA in a buffer (10 mM Tris-HCl, pH 7.6, 2 mM $CaCl_2$ and 2 mM $MgCl_2$) for various periods of time. The DNA was extracted and subjected to electrophoresis on 1.6% agarose gel. (1) Molecular size marker, (2) control calf thymus DNA, (3) 15 min, (4) 30 min, (5) 60 min, and (6) 120 min incubation.

Role of endonuclease activation in antimycin A-induced cell death

We next examined the effect of endonuclease inhibitors on antimycin A-induced cell death. Aurintricarboxylic acid provided complete protection against antimycin A-induced cell death as measured by trypan blue exclusion (Fig. 7A) and by LDH release (Fig. 7B), whereas fuchsin acid was without effect. Evans blue also provided significant protection against antimycin A-induced cell death as measured by trypan blue exclusion (Fig. 7C) and LDH release (Fig. 7D). Zinc sulfate (50 μM) provided significant but partial protection against antimycin A-induced cell death at three hours as measured by trypan blue exclusion (antimycin A alone, 35 ± 1% vs. antimycin A plus zinc, 23 ± 1%, $N = 3$, $P < 0.05$). Taken together, these data provide strong evidence for the presence of endonuclease activity and its important role in chemical hypoxia-induced DNA damage and cell death in LLC-PK$_1$ cells.

Discussion

Several recent *in vivo* studies suggest endonuclease activation in ischemic injury. For example, DNA fragmentation in the kidney cortex was detected 12 hours after reperfusion injury [22]. Other preliminary studies recently reported support the role of endonuclease activation in ischemic injury [23, 24]. Nogae et al, recently reported that cells undergoing apoptosis were identified immunohistochemically by labeling the 3′-OH DNA ends generated by DNA fragmentation after subjecting kidneys to ischemia/reperfusion [24]. The typical ladder consisting of approximately 200 bp was reported to be present in DNA isolated from ischemia/reperfusion model but not from sham operated controls. Although these studies demonstrate that this form of DNA damage may occur *in vivo*, these data do not show whether DNA fragmentation is an early event prior to loss of cell viability and the potential role of endonuclease activation in cell death.

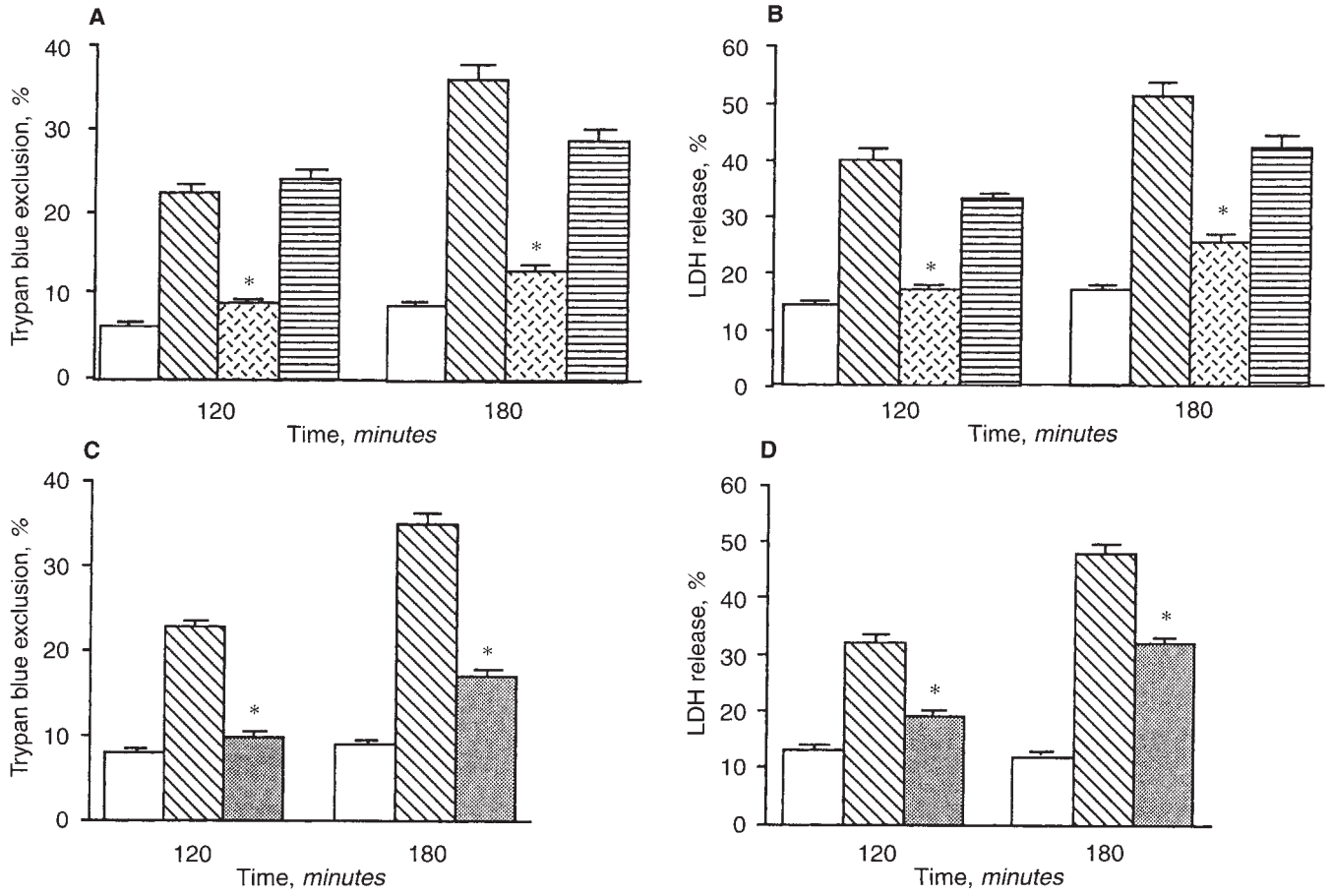

Fig. 7. *Effect of an endonuclease inhibitor, aurintricarboxylic acid on antimycin A-induced cytotoxicity in LLC-PK$_1$ cells.* **A.** Cells were exposed to antimycin A (10 μM) for 120 and 180 minutes in the absence or presence of aurintricarboxylic acid (10 μM) or fuchsin acid (50 μM), a structural analog of aurintricarboxylic acid. Cell death was measured by trypan blue exclusion. Results are means ± SE, $N = 5$ to 7. $^*P < 0.0001$, compared to cells exposed to antimycin A alone. **B.** Cell death was measured by LDH release. Results are means ± SE, $N = 3$. $^*P < 0.005$ compared to cells exposed to antimycin A alone. Symbols are: control (□), antimycin A alone (▨), antimycin A plus aurintricarboxylic acid (▩), and antimycin A plus fuchsin acid (▤). **C.** Effect of an endonuclease inhibitor, Evans blue on antimycin A-induced cytotoxicity in LLC-PK$_1$ cells. Cells were exposed to antimycin A (10 μM) in the absence or presence of Evans blue (2 μg/ml) for 120 and 180 minutes. Cell death was measured by trypan blue exclusion. Results are means ± SE, $N = 5$. $^*P < 0.001$, compared to cells exposed to antimycin A alone. **D.** Cell death was measured by LDH release. Results are means ± SE, $N = 3$ to 4. $^*P < 0.05$, compared to cells exposed to antimycin A alone. Shown are control (□), antimycin A alone (▨), antimycin A plus Evans blue (▤).

In the present study, we have shown that subjecting LLC-PK$_1$ cells to chemical hypoxia causes DNA damage that clearly precedes cell death. This is in contrast to a recent study in which it was concluded that the DNA damage was a result of necrotic cell death [25]. We provide substantive evidence that the DNA damage is the result of endonuclease activation. Incubating LLC-PK$_1$ cells with antimycin A resulted in DNA fragments in multimers of 180 bp, a characteristic feature of endonuclease induced DNA damage, rather than a necrotic form of cell death which typically results in a smear pattern on agarose gel. We prepared our samples under conditions that did not result in DNA fragmentation during tissue preparation [26]. In addition to the demonstration of DNA fragmentation, we have shown that incubating protein extract of cells subjected to chemical hypoxia with calf thymus DNA resulted in oligonucleosome length fragments, an effect that was prevented by an endonuclease inhibitor, aurintricarboxylic acid. Protein extract of cells subjected to chemical hypoxia also demonstrated increased DNA-degrading activity on a substrate gel. Endonuclease inhibitors provided almost complete protection against DNA damage. Taken together, these data indicate that endonuclease activation is an early event in chemical hypoxia and causes DNA damage.

It is known that in certain cell types DNA damage provokes rapid suicide [27]. For reasons that are not known, certain cell types in response to endonuclease-mediated DNA damage more regularly initiate a cascade of events that lead to cell death. In other cell types this appears to depend on the type and duration of the insult. The importance of the endonuclease activation in DNA damage and cell death was examined by utilizing endonuclease inhibitors. Endonuclease inhibitors provided protection against DNA damage and cell death, indicating that endonuclease activation did indeed contribute to cell death. Thus, *in vivo* data, data utilizing freshly isolated tubules, as well as cultured cells support the role of endonuclease in hypoxic injury. Currently, there is no information whether or not endonuclease inhibitors prevent DNA damage and cell death in *in vivo* models of renal injury. However, results from other tissues [28] suggest the possibility that endonuclease inhibitors may prevent renal injury.

Acknowledgments

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