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Effect of pyruvate on oxidant injury to isolated and cellular DNA

KARL A. NATH, HELEN ENRIGHT, LOUISE NUTTER, MICHAEL FISCHEREDER, JING-NAN ZOU, and ROBERT P. HEBBEL

Departments of Medicine and Pharmacology, University of Minnesota School of Medicine, Minneapolis, Minnesota, USA

Effect of pyruvate on oxidant injury to isolated and cellular DNA. Drawing upon the capacity of pyruvate to detoxify H_2O_2, we demonstrate that pyruvate (i) protects against H_2O_2-dependent, hydroxyl radical-mediated degradation of isolated DNA; (ii) reduces the amount of 8-hydroxy-2-deoxyguanosine detected following oxidative injury to isolated DNA and (iii) diminishes the amounts of detectable hydroxyl radical generated by a H_2O_2-dependent system. Compared to mannitol, pyruvate protects weakly against oxidative degradation of DNA induced by a H_2O_2-independent, hydroxyl radical-generating system. The protective effects of pyruvate against H_2O_2-instigated DNA damage were also evinced in cells in culture exposed to H_2O_2. In contrast to its protective effects against H_2O_2-dependent injury to DNA, pyruvate failed to offer convincing protection to another intracellular, H_2O_2-vulnerable target, glyceraldehyde-3-phosphate dehydrogenase. The protection conferred by pyruvate to intracellular H_2O_2-vulnerable targets is thus influenced by the nature of the target exposed to H_2O_2. Pyruvate was markedly protective in a model of cytotoxicity induced by the concomitant depletion of cellular glutathione and inhibition of catalase activity; pyruvate can thus function as an intracellular antioxidant and in this latter model, no evidence of DNA damage was observed. Pyruvate, in contrast to catalase, is a potent protector against cytotoxicity induced by organic peroxides, a finding that cannot be explained by the scavenging of organic peroxides, differences in glutathione content or attenuation in oxidative injury to DNA. We conclude that while DNA damage is a key pathogenetic event in oxidative stress induced by H_2O_2, such nuclear changes may not universally subserve a critical role in models of H_2O_2-dependent cell death. We also conclude that the antioxidant capabilities of pyruvate extend beyond scavenging of H_2O_2 to include potent protection against cytotoxicity induced by organic peroxides.

Inordinate generation of hydrogen peroxide (H_2O_2) either from infiltrating, inflammatory cells or cells indigenous to organs and tissues, is implicated in the pathogenesis of tissue injury and dysfunction of the kidney and other organs [1, 2]. The cellular defense mounted against such excessive generation of H_2O_2 resides in the enzymes, glutathione peroxidase and catalase [1, 2]. Recently, we have directed attention to the antioxidant properties of pyruvate and other members of the alpha-ketoacid family [3, 4]. In the presence of H_2O_2, these metabolites undergo oxidative decarboxylation while the oxidant is detoxified to water [3]. This chemical property is functionally significant since pyruvate protects against injury to renal epithelial cells *in vitro* induced by reagent H_2O_2 or enzymatically generated H_2O_2 [3, 4]. In addition to its relatively nontoxic nature, the virtue of pyruvate as a potentially clinically useful antioxidant resides in its ready transmissibility across plasma membranes and its unimpeded dissemination within the intracellular compartment [5], properties that ensure delivery of this metabolite to sites where oxidants are generated. Interestingly, other members of the alpha-ketoacid family are protective against functional decline in chronic renal insufficiency [6], a circumstance in which oxidative stress has been incriminated in the pathogenesis of tissue injury [7].

Multiple cellular organelles and molecular domains are increasingly identified as targets for H_2O_2-instigated injury [8, 9]. One of the earliest insults sustained by cells exposed to H_2O_2 is nuclear damage which, in turn, entrains a cascade of events that culminates in cell death [8, 9]. For example, within seconds of exposure of murine macrophages to H_2O_2, single strand breaks in DNA appear, leading to the activation of the DNA repair enzyme, poly-ADP-ribose polymerase. Such enhanced enzyme activity avidly utilizes NAD+, thereby impairing ATP synthesis and, by compromising cellular supplies of energy, presages cell death [8, 9]. Several candidates are incriminated in the process of DNA strand breakage in cells exposed to H_2O_2 or H_2O_2-generating systems including the highly reactive hydroxyl radical. For example, redox-cycling quinones provoke hydroxyl radical generation and DNA breaks in human breast carcinoma cells in culture, while concomitant treatment with catalase decreases the generation of hydroxyl radical, attenuates DNA damage and improves clonogenic survival of these cells [10]. Such production of hydroxyl radicals, in all likelihood, arises from the Fenton reaction catalyzed by transition metals such as iron or copper that are either bound to DNA or released from neighboring cellular sites following exposure to oxidants [11]. Oxidant-triggered DNA damage may also be mediated through the digestive effect of endonucleases [12]. For example, as recently demonstrated by Ueda and Shah, LLC-PK_1 cells, a cell line derived from the porcine renal proximal tubule, when exposed to H_2O_2, display activation of endonucleases, scission of DNA, and ultimately, cell death [12].

In light of such mounting importance assigned to DNA damage in the pathogenesis of oxidant-mediated cell death coupled to the potential clinical utility of pyruvate as an antioxidant, we examined the capacity of pyruvate to protect

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against DNA injury fomented by H_2O_2. We employed indices that encompass the ambit of alterations in DNA inflicted by oxidative insults. Oxidative injury, inducing single strand breaks to DNA in cultured cells, can be quantitated by the fluorescence alkaline unwinding technique [8, 13], whereas progressive scission of both DNA strands produces DNA fragments of diminishing molecular weight and which can be qualitatively assessed by gel electrophoresis [12, 14]. DNA fragmentation may also be assessed by the specific release of tritiated thymidine previously bound to DNA [12, 15]. Oxidative injury may be directed to a given base with attendant base change as, for example, the production of 8-hydroxy-2-deoxyguanosine (8OHdG) from the nucleoside, deoxyguanosine [16]. This latter change is potentially mutagenic since misreading at this altered base or adjacent bases may occur during subsequent DNA replication [17, 18].

Using this spectrum of indices, we examined oxidant-induced damage to isolated DNA as well as damage to DNA in cultured cells. We also examined the effect of pyruvate to protect against oxidative degradation of DNA induced by a H_2O_2-independent, hydroxyl radical-generating system. To induce oxidative stress we employed reagent H_2O_2, and a novel model of increased intracellular generation of H_2O_2, the latter based on depletion of intracellular glutathione [19, 20] combined with inhibition of catalase. To evaluate the efficacy of pyruvate in protecting against oxidative injury to DNA, we also examined the effect of pyruvate on injury inflicted by H_2O_2 on another vulnerable intracellular target, the thiol-containing enzyme, glyceraldehyde-3-phosphate dehydrogenase [21]. We also examined the effect of pyruvate on cellular injury induced by the organic peroxide, tert-butyl hydroperoxide [22].

Methods

Oxidant-induced DNA double strand breakage: Inhibition by pyruvate

The model of oxidative injury to isolated DNA was previously described by Enright, Miller and Hebbel [14]. Calf thymus DNA (60 μg) was exposed to an oxygen-radical generating system consisting either of H_2O_2 (500 μM), Fe^{2+}(20 μM)/ADP(40 μM) and ascorbate (500 μM) or of H_2O_2 (100 μM), Fe^{2+}(1 μM)/EDTA(2 μM) and ascorbate (250 μM) for one hour at 37°C. Differing concentrations of iron-EDTA and iron-ADP were used, since on a molar basis, iron-EDTA is a more efficient generator of the hydroxyl radical when compared to iron-ADP, although iron-ADP may be a more physiologically relevant intracellular iron chelate. Incubations were performed under iron-free conditions except when iron was deliberately added; solutions were rendered iron-free by treatment with Chelex resin. Pyruvate, in concentrations ranging from 0.25 to 5 mM, was present in some incubations. DNA damage in the form of double strand breaks was assessed by agarose gel electrophoresis [14].

Formation of 8OHdG: Inhibition by pyruvate

The formation of 8OHdG in DNA was measured as described by Floyd et al [16]. This oxidative index was measured in isolated DNA exposed to an oxygen radical generating system as well as in DNA extracted from cells exposed to oxidative stress induced by 1-chloro-2,4-dinitrobenzene, (CDNB) 250 μM, and aminotriazole, (AT) 10 mM, in the absence or presence of pyruvate. This model is based on depletion of intracellular glutathione by CDNB and the concomitant inhibition of catalase by AT. This combined treatment thus paralyzes the intracellular mechanisms that detoxify H_2O_2 generated during cellular metabolism. The CDNB was dissolved in ethanol and the final concentration of ethanol to which the cells were exposed was 0.5%. The same concentration of ethanol was added to the control wells. Such incubations were performed in the presence and absence of 2 mM pyruvate for four hours at 37°C. Following exposure to oxidant, LLC-PK_1 cells were scraped off and digested using Proteinase K (100 μg/ml; Bethesda Research Laboratories, Bethesda, Maryland, USA). DNA was extracted using phenol and chloroform, ethanol-precipitated, resuspended in 10 mM Tris/1 mM EDTA, pH 7.4 and extensively digested with the following enzymes as previously described [23]: DNAse 1 (bovine pancreas; Calbiochem, La Jolla; California, USA), endonuclease *Neurospora crassa* (Boehringer Mannheim, Mannheim, Germany), phosphodiesterase snake venom *Crotalus duriss* (Boehringer Mannheim) and alkaline phosphatase (calf intestine; Calbiochem). The amounts of deoxyguanosine (dG) and of 8OHdG in the resulting deoxynucleoside mixture were detected using a high performance liquid chromatograph (Hewlett Packard 1090LC) with in-line ultraviolet and electrochemical (Hewlett Packard EC 1049A) detectors, respectively. The analytical column used was a C18 5 μM ODS Hypersil 2.1 × 100 mm column (Hewlett-Packard #799160D-522 EA). The mobile phase was 2.8% methanol containing 4 mM citric acid, 8 mM sodium acetate (anhydrous), 10 mM sodium hydroxide, 0.067% glacial acetic acid, flow rate 0.3 ml/min. Results are expressed as $8OHdG/10^5dG$. The 8OHdG standard was a gift from Dr. Robert Floyd and the dG standard was purchased from Sigma Chemical Company (St. Louis, Missouri, USA).

Hydroxyl radical generation by iron-EDTA: Inhibition by pyruvate

The iron-EDTA-catalyzed hydroxyl radical generation from glucose/glucose oxidase (with ascorbate added as a reducing agent) was measured using dimethylsulfoxide, a hydroxyl radical scavenger which generates methane [24, 25]. All solutions were prepared in iron-free phosphate buffer (5 mM sodium phosphate/80 mM NaCl, pH 7.2). Iron-EDTA (1 μM ferrous iron/2 μM EDTA), glucose (30 mM), glucose oxidase (0.2 U/ml) and ascorbate (500 μM) were incubated for one hour at 37°C in the presence of increasing amounts of pyruvate (1.0, 2.0 and 5.0 mM). Headspace methane was then measured by gas chromatography [24, 25] using a Hewlett-Packard 5890A gas chromatograph (Hewlett-Packard Company, Avondale, Pennsylvania, USA) having a carbosieve B 100/120 mesh column and flame ionization detector. Methane was measured in parts per million (ppm) and converted to pmoles of hydroxyl radical generated, based on methane calibration gas standards (Scotty Analyzed Gases, ALLtech, Deerfield, Illinois, USA) and the 1:1 molar ratio of methane to hydroxyl radical.

Hydroxyl-radical dependent injury to DNA induced by radiolysis of water

We induced injury to DNA by a hydroxyl radical generating system wherein this latter species is produced independently of

H_2O_2 and the Fenton reaction. The radiolysis of water generates hydroxyl radical by a H_2O_2-independent pathway [26, 27] schematized as follows:

$$H_2O \rightarrow \cdot OH + e^-_{(aq)} + \cdot H$$

All solutions were rendered iron-free using iminodiacetic acid chelex resin prior to exposure to radiation. DNA was dissolved in a 5 mM phosphate buffer (100 μg/ml), pH 7.2 and exposed at room temperature to gamma-radiation (20,000 rads) using a ^{137}Cesium source. Studies were also carried out in the presence of mannitol (5 mM) or pyruvate (5 mM). The DNA was precipitated and then electrophoresed on a 0.8% agarose gel.

Measurement of single strand DNA breaks in LLC-PK$_1$ cells in vitro

We employed the method of Birnboim and Jevcak [13] as adapted by Schraufstatter et al [8]. This method is based on the selective binding of the fluorescent dye ethidium bromide to double-stranded DNA in preference to single-stranded DNA. The amount of fluorescence can be quantitated by spectrofluorometry, and thus provides a measure of percent double-stranded DNA remaining in cell lysates following exposure to oxidants in the presence or absence of pyruvate. In brief, 0.9 ml aliquots of LLC-PK$_1$ cells (in PBS at a concentration of 2.2×10^6 cells per ml) were exposed for 15 minutes at 37°C to H_2O_2 (500 μM) in the presence or absence of pyruvate (5 mM), then centrifuged, placed on ice and resuspended in 2 ml of a solution containing 250 mM mesoinositol, 10 mM sodium phosphate, 1 mM magnesium chloride, pH 7.2. The remaining steps are as described by Birnboim and Jevcak [13] with the exception that the final alkaline incubation at 15°C was for 30 minutes duration. Fluorescence was measured using a Perkins-Elmer LS-5B Luminescence Spectrometer (Norwalk, Connecticut, USA) with emission at 590 and excitation wavelengths at 520 nm.

DNA fragmentation based on the release of [^{3}H]-thymidine

We quantitated DNA fragmentation in LLC-PK$_1$ cells following exposure to oxidants using the method of Duke, Chernenak and Cohen [15] as modified by Ueda and Shah [12]. LLC-PK$_1$ cells were grown to confluency in six well Costar plates. The cells were labelled for 16 hours with [^{3}H]-thymidine, (5 μCi/well). Following washing with Basal Medium Eagle (BME) and incubation on ice for 60 minutes in BME, the cells were exposed to H_2O_2 (1 mM) in the absence or in the presence of pyruvate (5 mM). Incubation was at 37°C for five hours. Cells exposed to BME alone served as a control for the H_2O_2-treated cells while cells exposed to BME and pyruvate (5 mM) served as the control for cells treated with H_2O_2 and pyruvate. These respective control values were subtracted from the experimental conditions. There was no difference in the percent release of [^{3}H]-thymidine in control cells in BME alone and in control cells exposed to pyruvate and BME. Studies of DNA fragmentation were also performed following exposure of cells to CDNB (250 μM) and AT (10 mM) in the absence and presence of pyruvate (2 mM), and following the exposure of cells to tert-butyl hydroperoxide (100 μM) in the absence and presence of pyruvate (5 mM).

Inhibition of glyceraldehyde-3-phosphate dehydrogenase by hydrogen peroxide: Effect of pyruvate

Glyceraldehyde-3-phosphate dehydrogenase was measured as described by Hyslop et al [21]. The enzyme was incubated for five minutes with varying concentrations of H_2O_2. The protective of pyruvate (1 mM) was compared with that of catalase (800 U/ml) when either scavenger was incubated simultaneously with H_2O_2 (50 μM) and the enzyme. In addition we also tested the effect of H_2O_2 (50 μM) preincubated with pyruvate (1 mM) for five minutes prior to exposure of the enzyme to H_2O_2. Results are expressed as percent control enzyme activity. Pyruvate *per se* exerted a weak inhibitory effect on the reaction employed to assay enzyme activity; enzyme activity was decreased by 22% in the presence of pyruvate (1 mM). Thus, the results for inhibition of enzyme activity by H_2O_2 in the presence of pyruvate are expressed as a percent control enzyme activity when the enzyme is exposed to pyruvate alone.

Model of cytotoxicity induced by CDNB and AT

This model is based on depletion of intracellular glutathione by CDNB and the concomitant inhibition of catalase by AT. this combined treatment thus paralyzes the intracellular mechanisms that detoxify H_2O_2 generated during cellular metabolism. To determine the toxicity induced by such exposure, we employed the cytotoxicity assay based on the percent specific cytolytic release of ^{51}-Chromium [4]. Briefly, cells were loaded with 2 μCi of sodium ^{51}-Chromate in Hank's buffered salt solution (HBSS) for three hours at 37°C. The cells were washed and then incubated in BME in the presence and absence of CDNB (250 μM) and AT (10 mM).

Model of cytotoxicity induced by tert-butylhydroperoxide

We also examined an oxidant model of cytoxicity based on the exposure of LLC-PK$_1$ cells to the organic peroxide, tert-butyl hydroperoxide. Monolayers of cells were exposed to tert-butylhydroperoxide (100 μM) alone, tert-butylhydroperoxide (100 μM) and pyruvate (5 mM), and tert-butylhydroperoxide (100 μM) and catalase (800 U/ml) for six hours after which cytotoxicity was determined. In separate studies we examined cellular content of glutathione following such experimental conditions.

Determination of H_2O_2 concentration

We employed the phenol red assay as previously described for the measurement of H_2O_2 following the exposure of cells to H_2O_2 in the presence and absence of pyruvate [3]. We used the Xylenol Orange assay for a more sensitive determination of H_2O_2 in cytotoxcity studies employing CDNB and AT [28]. This assay measures H_2O_2 to nanomolar levels. This method is based on the oxidation of ferrous to ferric iron by H_2O_2. The ferric iron so formed is detected on the basis of a complex it forms with the dye Xylenol Orange that absorbs at 560 nm. The ferrous iron is prevented from autoxidation by dilute sulfuric acid. Sorbitol is used to amplify the colorimetric signal [28].

Detection of organic peroxides

Since organic peroxides also oxidize ferrous to ferric iron and such iron can be detected by the dye Xylenol Orange, this

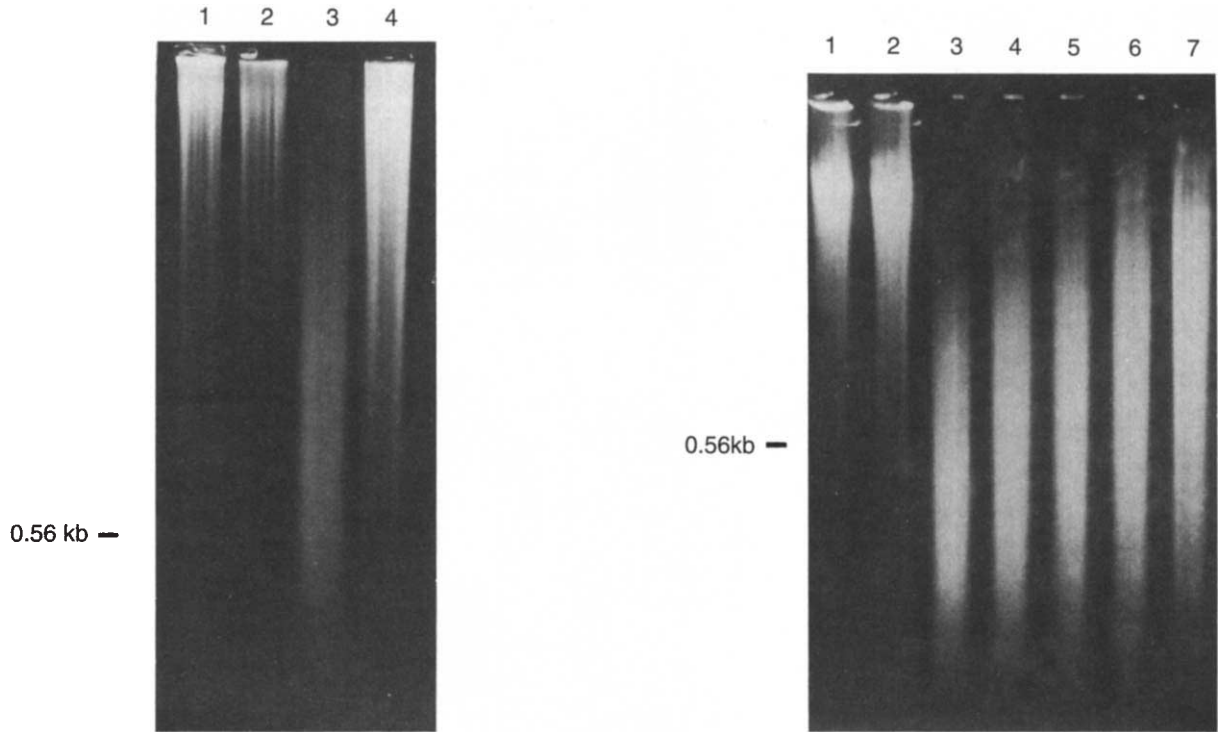

Fig. 1. Left panel A. *The effects of pyruvate on degradation of isolated DNA induced by oxidants.* The numbered lanes represent DNA alone (lane 1); DNA exposed to H_2O_2 (500 μM) + ascorbate (500 μM) (lane 2); DNA + H_2O_2 (500 μM) + iron/ADP (20 μM/40 μM) + ascorbate (500 μM) (lane 3); DNA + H_2O_2 (500 μM) + iron/ADP (20 μM/40 μM) + ascorbate (500 μM) + pyruvate (5 mM) (lane 4). Electrophoresis was on a 0.8% agarose gel (10 μg DNA/lane). **Right panel B.** Dose-dependent effect of pyruvate on degradation of isolated DNA induced by oxidants. The numbered lanes represent DNA alone (lane 1); DNA exposed to H_2O_2 (100 μM) + ascorbate (250 μM) (lane 2); DNA + H_2O_2 (100 μM)+ iron/EDTA (1 μM/2 μM) + ascorbate (250 μM) (lane 3); DNA + H_2O_2 (100 μM) + iron/EDTA (1 μM/2 μM) + ascorbate (250 μM) + increasing concentrations of pyruvate (0.25 mM, lane 4: 0.5 mM, lane 5: 1 mM, lane 6: 2 mM, lane 7). Electrophoresis was on a 0.8% agarose gel (10 μg DNA/lane).

assay, performed in the presence of pyruvate, can be used to determine whether pyruvate scavenges organic peroxides and/or prevent oxidation of iron by peroxides. We thus determined A560 values obtained with increasing concentrations of tert-butyl hydroperoxide in the absence and presence of pyruvate.

Measurement of total cellular thiol content

We utilized the method based on the reduction of the Ellman reagent, 5 5′-dithiobis(2-nitrobenzoic acid) [29]. LLC-PK_1 were grown to confluence on 10 cm petri plates. They were incubated with PBSG in the absence or presence of CDNB (250 μM) and AT (10 mM). Pyruvate (3 mM) was added to half the wells in either group. Following incubation for four hours at 37°C, the cells were washed, trypsinized, lysed and an aliquot of the supernatant added to 5 5′-dithiobis (2-nitrobenzoic acid), 40 mg/dl in 1% sodium citrate. The absorbance at 412 nm was determined and the results expressed as total cellular thiol content, nmol/mg protein.

Interactive laser cytometry

We determined hydrogen peroxide-dependent cellular fluorescence in cells exposed to oxidative insults in the presence or absence of pyruvate using interactive laser cytometry and the hydrogen peroxide sensitive dye, 2′7′-dichlorofluorescein diacetate by methods fully described previously [4].

Statistics

For statistical comparisons involving more than two groups, ANOVA and Neuman-Keuls test were performed using the Clinfo Statistical Program (BBN Software Products, Cambridge, Masschusetts, USA). For comparisons involving two groups the unpaired t-test was employed. Differences were considered significant for $P < 0.05$.

Results

Effect of pyruvate on oxidant-induced DNA damage

We induced extensive DNA degradation by an oxygen radical generating system consisting of H_2O_2 (500 μM), iron/ADP (20 μM ferrous iron and 40 μM ADP) and a reducing agent (ascorbate, 500 μM). As shown in the representative electrophoretic gel in Figure 1A, this system induced extensive DNA degradation when all components of the free radical generating system were present (lane 3). The addition of pyruvate (5 mM) markedly inhibited DNA fragmentation as reflected by diminished amounts of low molecular weight fragments (lane 4). Similar double strand cleavage was produced using an alternative iron chelate (iron/EDTA) ascorbate and hydrogen peroxide (Fig. 1B). The inhibitory effects of pyruvate were evinced in a dose-dependent fashion as shown in Figure 1B. With increasing concentrations of pyruvate 0.25 mM, 0.5 mM, 1 mM, and to a

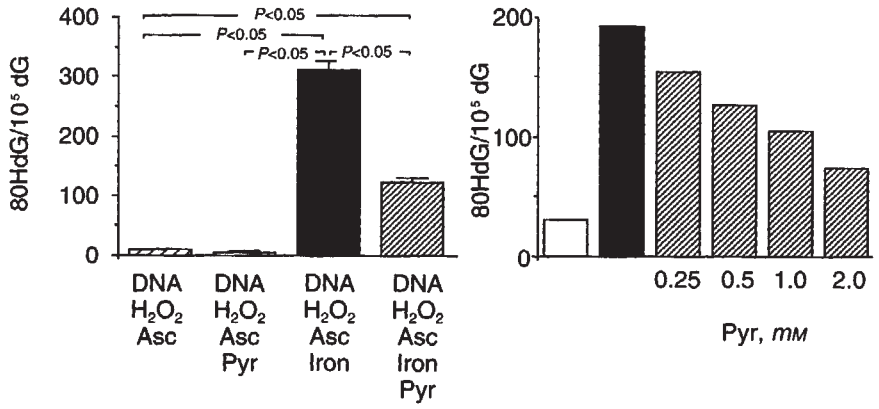

Fig. 2. A. *Effect of pyruvate (5 mM) on the generation of 8-hydroxy-2-deoxyguanosine following oxidative degradation of isolated DNA by H_2O_2 (500 μM), iron/ADP (20 μM/40 μM) and ascorbate (500 μM).* All conditions were performed in quadruplicate except DNA/H2O2/Pyr which was performed in triplicate. Data are Means ± SEM. **B.** Dose-dependent effect of pyruvate in the generation of 8-hydroxy-2-deoxyguanosine following oxidative degradation of isolated DNA by H_2O_2 (100 μM), iron/EDTA (1 μM/2 μM) and ascorbate (250 μM). Each condition was performed in duplicate.

maximum concentration of 2 mM, there was a step-wise decrease in double strand breaks induced by the oxygen radical generating system. We confirmed that the incremental addition of pyruvate to DNA did not influence the electrophoretic mobility of DNA (data not shown).

Using an oxygen radical generating system consisting of H_2O_2, iron-ADP and ascorbate, we demonstrated marked oxidative injury to DNA as indicated by oxidative base change (the generation of the oxidant marker, 8OHdG from dG; Fig. 2A). In the absence of iron virtually no damage to DNA was evident. Pyruvate (5 mM) inhibited the generation of 8OHdG; amounts of 8OHdG detected following the exposure of isolated DNA to the free radical generating system with added pyruvate were approximately 40% of the quantities detected in identical conditions in the absence of pyruvate. Such protective effects of pyruvate were evinced in a dose-dependent manner as revealed in Figure 2, right panel. Increasing amounts of pyruvate led to a step-wise decrement in the amounts of 8OHdG generated in DNA.

Generation of the hydroxyl radical: Effect of pyruvate

Since H_2O_2 and iron, under reducing conditions, generate the hydroxyl radical, and this latter reactive oxygen species accounts for the direct damaging effects to DNA [11], we determined whether the presence of increasing amounts of pyruvate would decrease the amount of hydroxyl radical detected under these conditions. As shown in Figure 3, with increasing amounts of pyruvate (1, 2, and 5 mM), there was a step-wise decrement in the amounts of hydroxyl radical detected in a system consisting of glucose/glucose oxidase (a source of H_2O_2, and thus an H_2O_2-*dependent* system), ascorbate and iron-EDTA.

We also determined whether pyruvate would decrease the oxidative degradation of DNA induced by hydroxyl radical, the latter generated by an H_2O_2-*independent* mechanism. Shown in Figure 4 is the extensive degradation of DNA provoked by hydroxyl radical generated by radiolysis (^{137}Cesium) of water. Mannitol, a potent scavenger of the hydroxyl radical, completely inhibited the degradation of DNA. Compared with mannitol, pyruvate weakly protected against the degradation of DNA induced by this system. These findings suggest that pyruvate is a weak scavenger of the hydroxyl radical, an interpretation consistent with the reported values for the rate

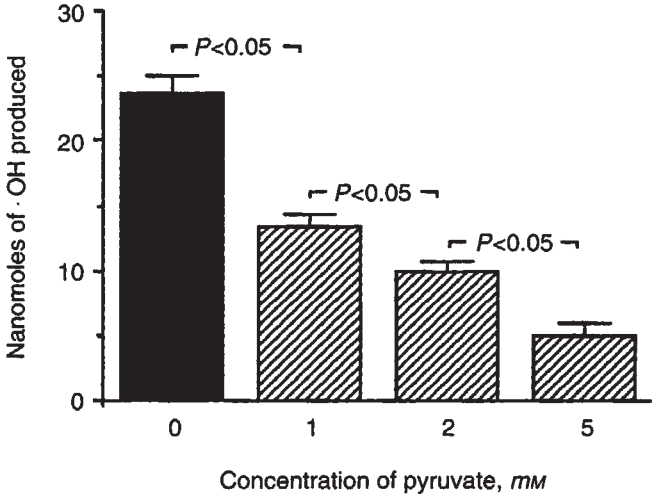

Fig. 3. *Dose-dependent effects of pyruvate on hydroxyl radical generation by iron-EDTA (1 μM/2 μM), glucose (30 mM)/glucose oxidase (0.2 U/ml) and ascorbate (500 μM).* Each condition was performed in quadriplicate. Data are means ± SEM.

constant of the reaction of pyruvate and the hydroxyl radical [27].

Protective effect of pyruvate on oxidant-induced DNA damage in LLC-PK$_1$ cells

Since pyruvate protected against injury to isolated DNA induced by oxidants *in vitro*, we questioned whether such injury to DNA in cells in culture exposed to oxidants would be attenuated by pyruvate. We measured oxidative injury of DNA using the single strand break assay. As shown in Figure 5A, exposure of LLC-PK$_1$ cells to H_2O_2 (500 μM) decreased the amount of double stranded DNA to approximately 40% of control cells, whereas in the presence of pyruvate, the percent double stranded DNA remaining at the end of the 15 minutes of incubation with H_2O_2 was significantly enhanced. Thus, the capacity of pyruvate to protect against oxidative injury to isolated DNA is coupled to such actions occurring *in vitro* in a cell culture system.

We also utilized the DNA fragmentation assay, as employed by Ueda and Shah [12], to quantitate DNA injury in cells

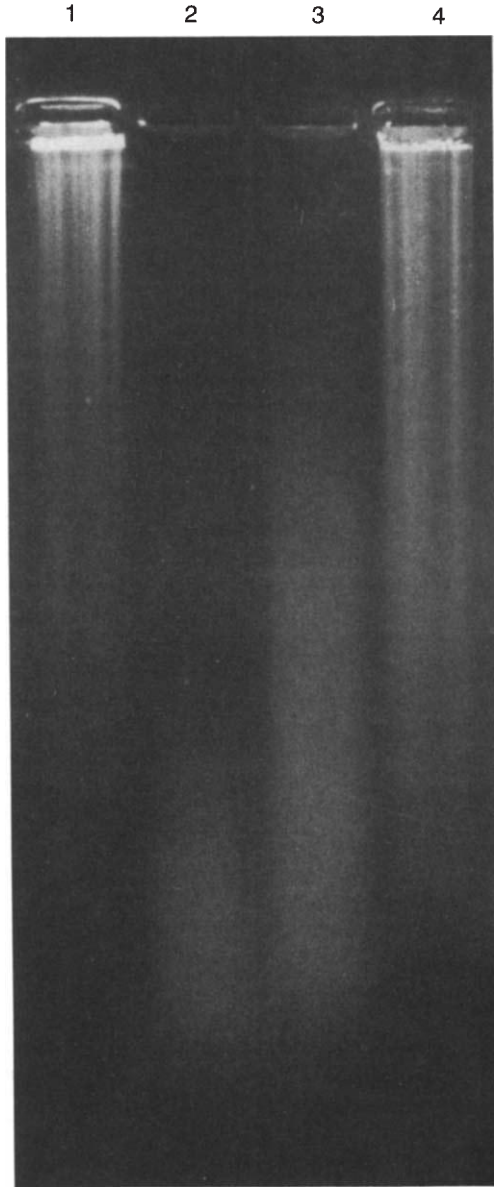

Fig. 4. *The effects of pyruvate on oxidative degradation of isolated DNA by ^{137}Cesium-induced radiolysis of water.* The numbered lanes represent DNA alone (lane 1); DNA exposed to 20,000 rads (lane 2); DNA exposed to 20,000 rads in the presence of pyruvate (5 mM) (lane 3); DNA exposed to 20,000 rads in the presence of mannitol (5 mM) (lane 4). Electrophoresis was on a 0.8% agarose gel (10 μg DNA/lane).

exposed to H_2O_2 *in vitro*. As shown in Figure 5B, H_2O_2 induced significant injury to DNA, as reflected by the specific release of [^{3}H] thymidine previously bound to DNA. Pyruvate again proved remarkably efficacious: DNA fragmentation was markedly and significantly reduced in the presence of pyruvate.

We examined whether pyruvate degraded hydrogen peroxide under conditions in which pyruvate attenuated cellular DNA injury as measured by the alkaline unwinding assay. We confirmed that the presence of pyruvate led to dose-dependent scavenging of H_2O_2, initially added at 500 μM to the extracellular medium. Indeed at concentations of pyruvate greater than 2 mM no H_2O_2 was detectable in the extracellular medium (data not shown).

Effect of pyruvate on oxidant-induced damage to other intracellular targets

In the light of the ability of pyruvate to protect against H_2O_2-dependent injury to isolated and cellular DNA, we examined the efficacy of such protection for another H_2O_2-vulnerable intracellular target. We chose the thiol-containing enzyme, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). As shown in Figure 6A, there was a dose-dependent reduction in GAPDH activity following exposure to H_2O_2. In Figure 6B, we assessed the relative protection provided to this enzyme by pyruvate (1 mM) or catalase when either scavenger was incubated simultaneously with H_2O_2 and the enzyme. The diminution in enzyme activity by H_2O_2 (50 μM) was not significantly different in the absence (Column #1) or presence (Column #2) of pyruvate. In contrast, catalase almost completely prevented the impairment in enzyme activity induced by H_2O_2 (Column #3). A protective effect of pyruvate, albeit not as great as the effect of catalase, was observed if H_2O_2 was incubated with pyruvate for five minutes *prior* to exposure of the enzyme to H_2O_2 (Column #4). ANOVA followed by the Neuman-Keuls test revealed that the mean represented by column 4 was significantly different from 1, 2 and 3 while the mean represented by column 3 was significantly different from 1, 2 and 4 (Fig. 6B).

A marked variation thus exists in the extent of protection conferred by pyruvate to H_2O_2-vulnerable targets, such as glyceraldehyde-3-phosphate dehydrogenase and DNA, when these targets are exposed to H_2O_2.

Influence of pyruvate on cell viability and DNA integrity of glutathione depleted cells

Under the conditions employed in our study, CDNB and AT induced marked cell injury. As shown in Figure 7A, treatment of cells with CDNB and AT induced almost 50% specific cytotoxicity. Such cell damage was markedly reduced in the presence of pyruvate. Cell damage induced by CDNB and AT was accompanied by a precipitous decline in total cellular thiol content; the addition of pyruvate did not further change total cellular thiol content (Figure 7B).

Treatment of cells with CDNB and AT provoked increased cellular content of H_2O_2 as reflected by greater amounts of H_2O_2 in the extracellular medium. The concentration of H_2O_2 was some 25-fold increased compared to cells exposed to buffer alone or to buffer containing pyruvate (Fig. 8). Pyruvate reduced such generation of H_2O_2, and indeed, the levels of H_2O_2 in the extracellular medium in the presence of pyruvate were not significantly different from those of controls. Thus, the cytoprotective effect of pyruvate was accompanied by reduced generation of H_2O_2 as reflected by levels of this oxidant in the extracellular space.

We also directly confirmed reduction in intracellular levels of hydrogen peroxide in this model following the administration of pyruvate. The increase in fluorescence displayed by cells due to exposure to CDNB and AT was significantly attenuated in the presence of pyruvate (221 ± 20 vs. 133 ± 14 Meridian Fluorescence Units, $P = 0.01$). Thus, CDNB and AT provoked

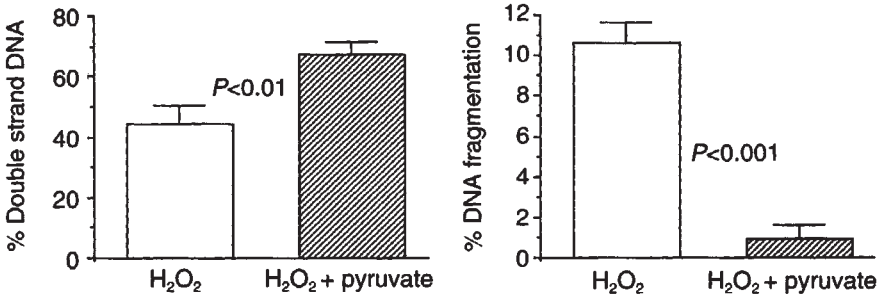

Fig. 5. A. *Effect of pyruvate (5 mM) on single strand breaks in DNA in LLC-PK$_1$ cells in vitro induced by H_2O_2 (500 μM).* The data represent the average of seven separate determinations. Data are means ± SEM. **B.** Effect of pyruvate on DNA fragmentation as measured by the specific release of [^{3}H] thymidine. Release of thymidine induced by exposure to H_2O_2 (1 mM), in the absence or presence of pyruvate (5 mM), was performed in 10 wells in either experimental condition. Data are means ± SEM.

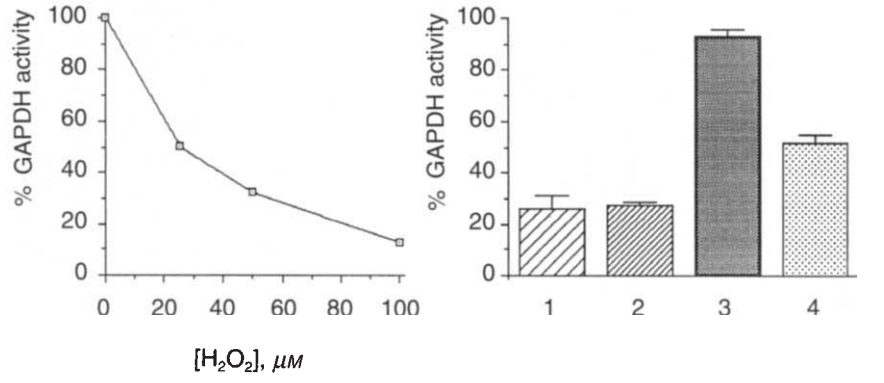

Fig. 6. A. *H_2O_2-mediated dose-dependent decrease in activity of glyceraldehyde-3-phosphate dehydrogenase.* Each value represents the means of duplicates. B. Effect of pyruvate and catalase on H_2O_2-induced impairment in activity of glyceraldehyde-3-phosphate dehydrogenase. The enzyme was exposed to: H_2O_2 (50 μM) alone (column 1), H_2O_2 (50 μM) and pyruvate (1 mM) simultaneously (column 2), H_2O_2 (50 μM) and catalase (800 U/ml) simultaneously (column 3), H_2O_2 (50 μM) that was pre-incubated with pyruvate (1 mM) for 5 minutes (column 4). Each condition was performed in quadriplicate. Data are means ± SEM. The mean represented by column 4 was significantly different than 1, 2 and 3 while the mean represented by column 3 was significantly different than 1, 2 and 4 by ANOVA.

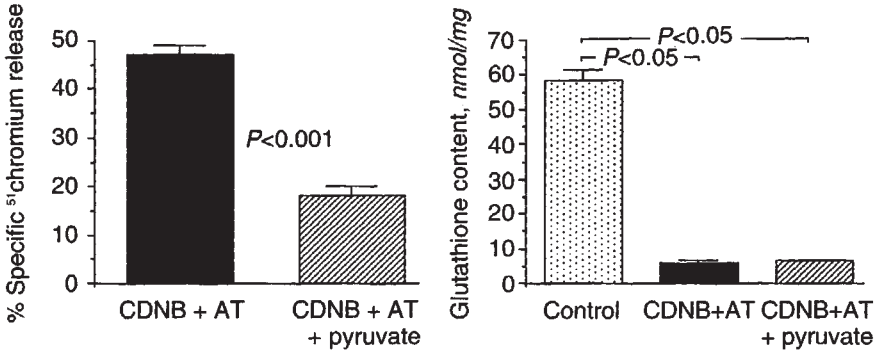

Fig. 7. A. *Effects of pyruvate (2 mM) on cytotoxicity induced by CDNB (250 μM) and AT (10 mM).* The means represent the average of seven separate assays, each performed in quadriplicate. **B.** Total cellular thiol content in LLC-PK$_1$ cells exposed to buffer alone, buffer + CDNB (250 μM) + AT (10 mM), buffer + CDNB (250 μM) + AT (10 mM) + Pyr (3 mM). Each experimental condition was performed in quadriplicate. Data are means ± SEM.

increased intracellular H_2O_2-dependent fluorescence, and pyruvate which is protective in this model, decreases such fluorescence.

In contrast to the prominent DNA damage induced by reagent H_2O_2, the exposure of LLC-PK$_1$ cells to CDNB and AT, a manipulation that induces fulminant cytolysis, failed to provoke DNA damage, as measured by the DNA fragmentation assay. The percent release of [^{3}H] thymidine by cells exposed to CDNB and AT in the absence and presence of pyruvate was not different from the percent spontaneous release of [^{3}H] thymidine by LLC-PK$_1$ cells not exposed to CDNB and AT.

Using this model of pyruvate-inhibitable, oxidant-induced cell injury, we did not observe oxidative injury to DNA as measured by the generation of 8OHdG in DNA extracted from LLC-PK$_1$ cells. Values for 8OHdG/10^{5}dG, the means of duplicate or triplicate determinations, were comparable for all four groups: control: 34; control and pyruvate: 29; CDNB and AT: 30; CDNB and AT and pyruvate: 32.

Influence of pyruvate on cell viability and DNA integrity in cells exposed to organic peroxides

As shown in Figure 9 tert-butylhydroperoxide (100 μM) led to significant cytotoxicity; pyruvate was remarkably effective in attenuating such toxicty while in contrast, catalase exacerbated toxicity induced by tert-butylhydroperoxide (Fig. 9). Using the method of detecting oxidative injury to DNA based on the percent release of [^{3}H] thymidine by cells, we found no evidence of DNA damage in this model under the conditions employed.

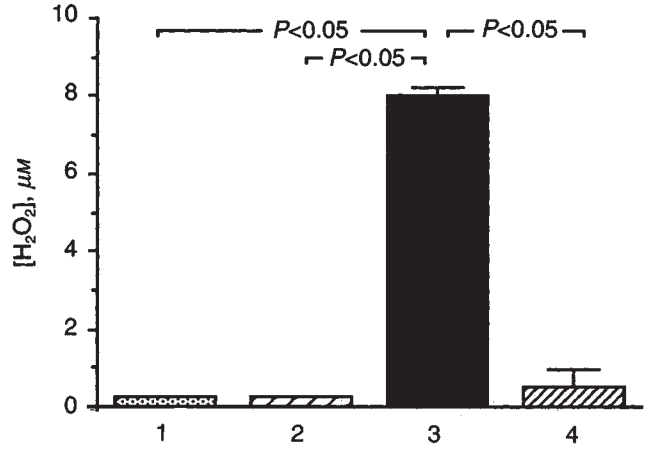

Fig. 8. *Effect of pyruvate on generation of H_2O_2 by cells treated with CDNB (250 μM) + AT (10 mM), as reflected by extracellular concentration of H_2O_2.* Controls in the absence or presence of pyruvate (2 mM) were studied in 4 wells while the effect of CDNB and AT in absence or presence of pyruvate were studied in 8 wells. Symbols are: (▨) column 1, HBSS; (▩) column 2, HBSS + Pyr; (■) column 3, HBSS + CDNB + AT; (▨) column 4, HBSS + CDNB + AT + Pyr. Data are means ± SEM.

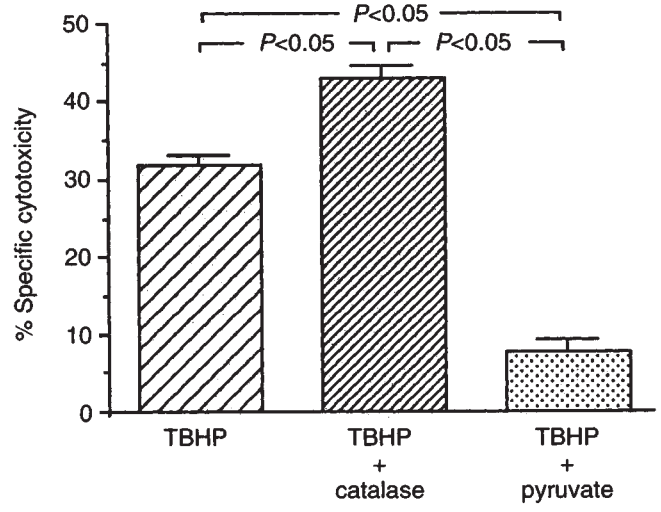

Fig. 9. *Effect of pyruvate (5 mM) and catalase (800 U/ml) on oxidant injury to LLC-PK_1 cells induced by tert-butyl hydroperoxide, TBHP, (100 μM) after exposure for 6 hours.* Each experimental condition represents the mean from 10 wells. Data are means ± SEM. All possible comparisons between groups 3 to 6 inclusive were significant by ANOVA.

We explored the mechanisms that may underlie the protective effects of pyruvate in this model. As shown in Table 1, neither the cytotoxicity of tert-butyl hydroperoxide nor the protective effect of pyruvate was accompanied by alterations in cellular content of glutathione. Using the Xylenol assay (which is based on peroxide-dependent oxidation of Fe^{2+}) pyruvate neither scavenges tert-butylhydroperoxide nor does pyruvate inhibit such oxidation of iron (Table 2).

DISCUSSION

Using multiple indices, we demonstrate that pyruvate attenuates oxidant-mediated DNA damage. Oxidative degradation of isolated DNA, as assessed by gel electrophoresis, was reduced in a dose-dependent fashion by pyruvate, as was the generation of 8OHdG. The protective effects of pyruvate extended to DNA injury in renal epithelial cells *in vitro* as revealed by a decrease in single strand DNA breaks and fragmentation of DNA, the latter quantitated by release of [^{3}H] thymidine when such cells are exposed to H_2O_2 in the presence of pyruvate. Thus, our data, in aggregate, attest to the efficacy of pyruvate as a protector against H_2O_2-mediated damage to isolated and cellular DNA.

We also devised a model of H_2O_2-induced stress originating in the intracellular compartment. Cellular glutathione was depleted by CDNB, an electrophilic agent that utilizes glutathione in generating thioether conjugates via the catalytic action of glutathione-S-transferase [19, 20]. Catalase was concomitantly inhibited by aminotriazole. So stripped of the pathways by which H_2O_2 is catabolized, lytic cellular injury occurred. While other cellular actions of this manipulation undoubtedly contribute to cytotoxicity, we confirmed that this manipulation is attended by increased amounts of H_2O_2 in the intracellular space and in extracellular medium. Pyruvate protected markedly against such cytotoxicity and concomitantly decreased concentrations of H_2O_2 in the intracellular and extracellular spaces. These findings demonstrate, for the first time, that pyruvate can rescue cells languishing under the exigency of having lost both intracellular mechanisms that scavenge H_2O_2. Endogenous intracellular stores of pyruvate may thus be considered, along with glutathione peroxidase and catalase, as part of the intracellular antioxidant defense system that regulates the prevailing levels of H_2O_2. Interestingly, this model of oxidative injury, one that provokes rapid and marked amounts of lytic cell death, failed to display DNA damage. Cell lysis in this circumstance is not a consequence of nuclear damage but, in all likelihood, reflects oxidant injury to non-nuclear targets as, for

Table 1. Cellular glutathione content of LLC-PK_1 cells (nmoles/mg protein) following exposure to tert-butyl hydroperoxide (100 μM) in the absence or presence of pyruvate

	3 Hour	6 Hour
Media alone	38.2 ± 0.3	36.5 ± 1.5
Media + tert-butyl hydroperoxide	34.7 ± 2.2	35.6 ± 1.6
Media + tert-butyl hydroperoxide + pyruvate	34.2 ± 0.6	41.9 ± 7.1

Concentrations of pyruvate employed in 3 and 6 hour incubations were 2 and 5 mM, respectively. Each value represents the mean of 4 or 5 determinations. No significant differences were observed in any of the comparisons.

Table 2. Effect of pyruvate (2 mM) on the detection of tert-butyl hydroperoxide by the Xylenol Orange assay

Tert-butyl hydroperoxide	A560 without pyruvate	A560 with pyruvate
3.13 μM	0.018	0.022
6.25	0.048	0.047
12.5	0.112	0.110
25.0	0.241	0.241
50.0	0.517	0.509
100.0	1.052	1.057

Each A560 reading was the mean of duplicates.

example, the plasma membrane, that are essential in the maintenance of cellular vitality.

We examined another model of oxidative stress, one based on the organic peroxide, tert-butyl hydroperoxide. Several mechanisms are implicated in cytotoxicity induced by this agent including glutathione depletion, lipid peroxidation, DNA injury and the influx of calcium [22, 30, 31]. Pyruvate markedly reduced cytotoxicity induced by tert-butyl hydroperoxide, a surprising finding especially since pyruvate and other alpha-ketoacids are not recognized as reactants with organic peroxides via the oxidative decarboxylation reaction, as occurs between alpha-ketoacids and hydrogen peroxide. To explain such protection we pursued several possibilities, all of which were found wanting. Pyruvate neither scavenges organic peroxide nor inhibit such oxidation of iron. Moreover, this model, al least under the conditions we employed, is not attended by DNA injury or depletion of glutathione, thus the beneficial actions of pyruvate cannot be attributed to attenuation of these biochemical lesions. This raises the possibility that other cellular actions of pyruvate may underlie its cytoprotection. Indeed we found that pyruvate can protect, albeit weakly, against hydroxyl radical-induced injury when the hydroxyl radical is generated independently of H_2O_2. Other actions of pyruvate that may contribute to its cytoprotection include its capacity to scavenge singlet oxygen [32] and its conversion to alanine, the latter amino acid exerting protective actions in vitro [33].

Interestingly, in stark contrast to pyruvate, catalase exacerbated tert-butyl hydroperoxide-induced cytotoxicity. We are unaware of any precedent in published studies of oxidant injury wherein such divergent effects of catalase and pyruvate occur. While the mechanisms by which catalase exacerbates such injury is beyond the scope of this study, we speculate that the heme-iron content of catalase may contribute to tert-butyl hydroperoxide-induced toxicity. Tert-butyl hydroperoxide dislodges iron from the heme ring of hemoglobin [34], and iron so released in turn catalyzes oxidative injury incited by tert-butyl hydroperoxide [35]. A similar mechanism may underlie the exacerbatory effects of other heme-proteins such as catalase.

Our studies reveal that intracellular H_2O_2-vulnerable targets may be quite heterogeneous with regard to the protection offered by pyruvate. While pyruvate efficiently protects against H_2O_2-induced DNA injury, the protection offered by pyruvate, as compared to catalase was minuscule when another oxidant-vulnerable cellular target, the thiol-containing enzyme, glyceraldehyde-3-phosphate dehydrogenase, was examined. This enzyme is enriched in cysteine residues and is quite sensitive to the oxidative effects of H_2O_2 [21]. Heterogeneity in protection, in all likelihood, is derived at least in part from the kinetic considerations underlying competing chemical interactions: faced with a choice of interacting with vulnerable thiol domains in an oxidant-sensitive enzyme on the one hand, and pyruvate on the other, hydrogen peroxide selects the thiol domain. The outcome is quite different when hydrogen peroxide is proffered DNA instead of the thiol-enzyme. In this circumstance, hydrogen peroxide reacts with alpha-ketoacid and dissipates itself, leaving DNA spared and free from oxidative degradation.

Our findings, highlighting the potent protective effects of pyruvate against oxidative injury to DNA, may be relevant to the mitochondrion, an organelle that accumulates pyruvate to millimolar quantities [36, 37]. While fulfilling its charge of providing the energy needs of the cell, the mitochondrial electron transport chain yields oxidant species [38]. That a certain fraction, albeit small (1 to 3%), of oxygen consumed is channeled, inexorably, to H_2O_2 presents a problem to the mitochondrion: housed within the mitochondrion are multiple potential targets for oxidants, including DNA. Moreover, mitochondrial DNA is devoid of histones [39], a class of proteins which confers resistance to nuclear DNA against oxidative attack [14]. Additionally, the mitochondrion as compared to the nucleus is relatively impoverished in DNA-repair enzymes [39]. Since mitochondria accumulate pyruvate to millimolar quantities [36, 37], we suggest that pyruvate, normally present within mitochondria, guards against the continual threat of oxidative injury to DNA posed by H_2O_2 issuing from the electron transport chain.

As demonstrated by Ueda and Shah, H_2O_2 triggers, quite early and prominently, endonuclease-dependent DNA damage and features of apoptotic cell death [12]. Programmed cell death, at least in some tissues, requires a relative preservation of the energy-generating capacity of the cell [40], the latter function subserved largely by the mitochondrion. Interestingly, mitochondrial constituents such as the *bcl* protein, may regulate the timing and occurrence of apoptosis [41]. Yet, in contrast to nuclear DNA, mitochondrial DNA does not undergo fragmentation during apoptosis [42]. In the light of the emerging perception of a role for oxidants in apoptosis, we speculate that the relative preservation of mitochondrial function in cells undergoing programmed death may reflect the copious amounts of mitochondrial pyruvate, the latter safeguarding mitochondrial DNA from oxidant damage.

We suggest that the efficacy of pyruvate in protecting against oxidative injury to DNA is particularly relevant to the widespread practice of adding pyruvate in millimolar quantities to cell culture media [43–45]. The rationale for such addition of pyruvate is based largely on the studies of Eagle and collaborators, performed some 30 years ago, which demonstrated the efficacy of pyruvate in promoting the proliferation of cells plated at low density [43, 44]. Cells are more vulnerable to nuclear injury during cell division. It is quite possible that the unremitting accumulation of oxidants, generated during cell proliferation may, ultimately, incur cytotoxicity. Since a healthy nuclear apparatus is critical to the process of normal cell proliferation, we speculate that the preservation of nuclear integrity by pyruvate against oxidants generated by respiring cells may underlie such salutary actions of pyruvate. A similar mechanism may account for the protection afforded by pyruvate and other alpha-ketoacids to mammalian cells in culture exposed to heat shock [46] since the latter insult can impose oxidative stress [47].

Our findings may be germane to oxidative stress in *in vivo* models. Oxidative injury to DNA occurs within one hour of the induction of iron-mediated acute renal failure *in vivo* [48, 49], and is present when measured 24 hours after halide-oxidant induced acute renal failure *in vivo* [50]. In such models, anti-oxidant maneuvers that are beneficial also reduce oxidative injury to DNA [51]. Oxidative injury to DNA may also be germane to chronic renal injury since dietary restriction, a maneuver that ameliorates renal injury [52] and reduces oxidative injury to DNA [53]. The effect of pyruvate on oxidant damage to DNA in the glycerol model of acute renal failure, one

in which pyruvate exerts a beneficial effect [3], would be of interest.

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Reprint requests to Karl A. Nath, M.D., Box 736 UMHC, 516 Delaware Street, SE, Minneapolis, Minnesota 55455, USA.

Appendix. Abbreviations

H_2O_2: hydrogen peroxide
CDNB: 1-chloro-2,4-dinitrobenzene
AT: aminotriazole
Asc: ascorbate
HBSS: Hanks balanced salt solution
BME: Basal Medium Eagle
PBSG: Phosphate buffered saline containing glucose
Pyr: Pyruvate
8OHdG: 8-hydroxy-2-deoxyguanosine
dG: 2-deoxyguanosine
TBHP: tert-butyl hydroperoxide
GAPDH: glyceraldehyde-3-phosphate dehydrogenase

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