Hydrogen Peroxide–induced Renal Injury

A Protective Role for Pyruvate In Vitro and In Vivo

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Abstract

Hydrogen peroxide (H_2O_2) contributes to renal cellular injury. α-Keto acids nonenzymatically reduce H_2O_2 to water while undergoing decarboxylation at the 1-carbon (1-C) position. We examined, in vitro and in vivo, the protective role of sodium pyruvate in H_2O_2-induced renal injury. Pyruvate effectively scavenged H_2O_2 in vitro, and suppressed H_2O_2-induced renal lipid peroxidation. Injury to LLC-PK$_1$ cells induced by hydrogen peroxide was attenuated by pyruvate to an extent comparable to that seen with catalase. Studies utilizing [1-^{14}C]pyruvate further demonstrated 1-C decarboxylation concurrent with cytoprotection by pyruvate from H_2O_2-induced injury. Pyruvate was also protective in vivo. Infusion of pyruvate before and during the intrarenal infusion of H_2O_2 attenuated H_2O_2-induced proteinuria. Systemic administration of pyruvate was also protective in the glycerol model of acute renal failure, a model also characterized by increased generation of H_2O_2. These findings indicate that pyruvate, a ubiquitous α-keto acid, scavenges H_2O_2 and protects renal tissue in vitro and in vivo from H_2O_2-mediated injury. These data suggest a potential therapeutic role for pyruvate in diseases in which increased generation of H_2O_2 is incriminated in renal damage. (*J. Clin. Invest.* 1991. 88:1886–1893.) Key words: α-keto acids • kidney • oxidant stress • proteinuria • scavenger

Introduction

Numerous studies have established an important role for reactive oxygen species in mediating injury in a variety of models of renal disease (1, 2). In particular, hydrogen peroxide (H_2O_2), a diffusible reactive oxygen metabolite formed by either enzyme-catalyzed or spontaneous dismutation of superoxide anion, is implicated in the pathogenesis of tissue damage in glycerol-induced injury (3), ischemia-reperfusion injury (4, 5), gentamicin nephropathy (6), and neutrophil-dependent glomerular injury (2, 7, 8). H_2O_2 is toxic when administered to the intact kidney (9) or the isolated perfused kidney (10), and inflicts cellular injury at multiple cellular sites either directly or through the generation of more reactive intermediates (11–14).

Hollemann (15) first described in 1904 the capacity of pyruvate and related α-keto acids to reduce H_2O_2 to water while these acids concomitantly undergo nonenzymatic decarboxylation at the one-carbon position (Fig. 1). Subsequent chemical studies have shown this reaction to be rapid and stoichiometric (16, 17). Yet surprisingly, very few studies utilizing mammalian cells, and certainly none employing kidney cells, have explored the capacity of pyruvate to protect against H_2O_2-induced injury (18–20). While demonstrating that pyruvate indeed protected against H_2O_2-induced injury in vitro, none of these studies examined the mechanism underlying such protective effects and specifically, whether such protective effects involved oxidative decarboxylation of pyruvate. Also unaddressed in any organ or tissue is whether this scavenging property allows protection against H_2O_2-induced injury in vivo.

In our study we confirmed the scavenging of H_2O_2 by pyruvate and provide the first demonstration that pyruvate protects against H_2O_2-induced injury in renal epithelial cells in vitro, and that such protection is accompanied by oxidative decarboxylation of pyruvate. Importantly, we also demonstrate that in two in vivo models characterized by H_2O_2-induced renal injury, the systemic administration of pyruvate protects against renal damage.

Methods

In vitro studies

Scavenging of H_2O_2 by pyruvate. The ability of pyruvate to scavenge H_2O_2 was studied by incubating the H_2O_2-generating systems, hypoxanthine/xanthine oxidase and glucose/glucose oxidase, in the presence and absence of pyruvate. Hypoxanthine, 5 mM, and xanthine oxidase, 40 mU/ml (Sigma Chemical Co., St. Louis, MO), were dissolved in Krebs-Ringer bicarbonate buffer with 100 mg/dl D-glucose and incubated for 3 h at 37°C in a shaking incubator. Incubations were performed in the presence and absence of 1.5 mM sodium pyruvate (Sigma Chemical Co.). Parallel studies were also performed with 0.15 U/ml glucose oxidase in Krebs-Ringer buffer with 100 mg/dl D-glucose. Aliquots from the reaction mixtures were assayed for H_2O_2 by the phenol red method of Pick and Keisari (21). Additional control studies failed to demonstrate significant inhibition of enzyme activity for xanthine oxidase, measured by uric acid generation or glucose oxidase, measured by glucose consumption.

Effect of pyruvate on H_2O_2-induced lipid peroxidation. We determined lipid peroxidation in rat whole-kidney homogenate by measuring thiobarbituric acid substances (TBARS)[1] using the method of Ohkawa et al. (22). Homogenates of kidney in 100 mM KCl were incubated at 37°C for 1 h in the presence of control buffer alone, buffer containing 4 mM H_2O_2, and buffer containing 4 mM H_2O_2 plus 4 mM

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1. *Abbreviations used in this paper:* BME, basal medium Eagle's; TBARS, thiobarbituric acid substances.

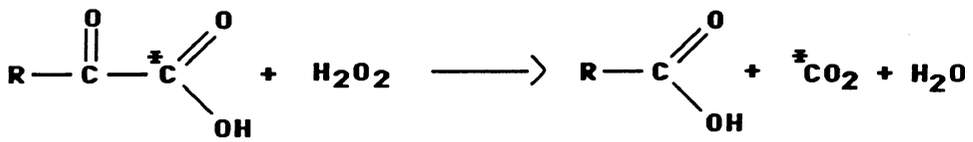

Figure 1. Decarboxylation of α-keto acids by H_2O_2.

sodium pyruvate. We chose this concentration of H_2O_2 since this amount has been shown previously to markedly induce lipid peroxidation in kidney tissue (23).

Effect of pyruvate on H_2O_2-induced injury to renal tubular epithelial cells in culture. LLC-PK_1 cells were obtained from the American Culture Collection, Rockville, MD, at passage no. 196. Cells were grown in Dulbecco's modified Eagle's medium (DME, Gibco Laboratories, Grand Island, NY) at 37°C in 95% air and 5% carbon dioxide. The medium contained 1 mM sodium pyruvate and was supplemented with 10% fetal calf serum, penicillin (100 U/ml), and streptomycin (100 μg/ml). Studies were performed on cultures over a range of no more than six passages. For experimental studies, cells were transferred to 24-well plates (Costar, Cambridge, MA) in DME and studied as a confluent layer. We quantified cell damage induced by H_2O_2 by determining cell lysis and detachment using the method of ^{51}Cr release (24).

LLC-PK_1 cells were radiolabeled by incubating each well with 2.0 μCi sodium [^{51}Cr]chromate (Amersham Corp., Arlinton Heights, IL) in 0.2 ml of Hank's buffered salt solution (HBSS, Gibco Laboratories) for 3 h at 37°C. Cells were then gently washed twice with 1.0 ml HBSS to remove excess radiolabel.

Immediately after ^{51}Cr loading, cells were placed in pyruvate-free basal medium Eagle's (BME) with 100 mg/dl glucose for incubation studies. To study the cytoprotective effect of graded concentrations of pyruvate in an H_2O_2-generating system, monolayers were incubated with 50 mU/ml xanthine oxidase and 5 mM hypoxanthine in the presence and absence of pyruvate for 3 h at 37°C in 95% air and 5% CO_2. Pyruvate was added in concentrations of 0.125, 0.250, 0.500, 1.0, and 2.0 mM. In separate experiments, the protective effect of 1.5 mM pyruvate against H_2O_2 generated by 40 mU/ml xanthine oxidase and 5 mM hypoxanthine was compared with that afforded by 1.5 mM malate (sodium malate), 100 μg/ml catalase and 100 μg/ml superoxide dismutase (SOD, all from Sigma Chemical Co). Malate, a tricarboxylic acid cycle intermediate, was used to control for the possibility of cytoprotection by pyruvate owing to pyruvate's serving as a metabolic fuel. Xanthine oxidase and hypoxanthine produce superoxide anion which spontaneously dismutates to H_2O_2. SOD was employed to assess any direct cytopathic role of superoxide on LLC-PK_1 cells. Additionally, cytoprotection by 1.5 mM pyruvate against H_2O_2 generated by glucose oxidase 0.15 U/ml in the presence of 100 mg/dl glucose was compared with that by 1.5 mM malate and 100 μg/ml catalase.

In separate studies we tested the capacity of equimolar amounts of sodium pyruvate to protect against injury to LLC-PK_1 cells induced by reagent hydrogen peroxide (1.5 mM). We also tested the effect of sodium pyruvate on nonoxidant injury to LLC-PK_1 cells induced by 0.1% Triton X-100.

After incubation studies BME was aspirated from each well and was combined with two 1.0-ml washings with HBSS. This was centrifuged and the supernatant (*A*), representing cytolytic release of ^{51}Cr, was counted (Gamma 7000, Beckman Instruments, Inc., Fullerton, CA) separately from the cell pellet (*B*), the later representing ^{51}Cr in detached cells. The remaining adherent cells (*C*) in each well were dissolved in 1.0 ml of 1.0 N sodium hydroxide and were counted separately. Adherent cell counts represented remaining viable cells. Percent cytolytic ^{51}Cr release was calculated by $\{A/(A + B + C)\} \times 100$ and percent detachment by $\{B/(A + B + C)\} \times 100$. Specific cytolytic release and detachment were calculated by subtracting the respective values obtained by incubating LLC-PK_1 in pyruvate-free BME alone.

Quantitation of pyruvate decarboxylation induced by H_2O_2. LLC-PK_1 were grown to confluence and loaded with sodium [^{51}Cr]-chromate. Cell monolayers were treated with trypsin followed by 10% fetal bovine serum and then gently washed twice in HBSS. ^{51}Cr-loaded cells were suspended at a concentration of 2.5×10^5/ml in pyruvate-free BME for incubation studies. ^{51}Cr-loaded cell suspensions were incubated for 3 h in a 37°C shaking incubator in the following media: pyruvate-free BME alone, pyruvate-free BME + 1.5 mM H_2O_2, pyruvate-free BME + 1.5 mM H_2O_2 + 1.5 mM [1-^{14}C]sodium pyruvate (Amersham Corp.), and lastly, pyruvate-free BME + 1.5 mM [1-^{14}C]-sodium pyruvate. Specific activity of [1-^{14}C]sodium pyruvate used in these studies was 165.8 μCi/mmol. Incubations were carried out in sealed tubes in which plastic wells (Kontes Co., Vineland, NJ) containing filter paper discs saturated with 2.0 M sodium hydroxide were suspended above the cell suspension. One-carbon decarboxylation of [1-^{14}C]pyruvate was quantitated by trapping of liberated $^{14}CO_2$ by the method of Constantopoulos and Barranger (25). Simultaneous determination of specific cytolytic ^{51}Cr release was also performed for each study group. Isotope studies were performed with β and γ counters (Beckman Instrument Co.).

In vivo studies

EFFECT OF PYRUVATE ON H_2O_2-INDUCED PROTEINURIA IN VIVO

We performed renal clearance studies of the left kidney under euvolemic conditions in rats anesthetized with an intraperitoneal injection of 5-*sec*-butyl-5-ethyl-2-thiobarbituric acid (Inactin, 100 mg/kg body wt, Byk-Gulden, Constance, FRG) as previously described (26). To replace surgical losses and maintain constancy of initial hematocrit, isooncotic rat plasma (1% body wt) was administered over 30 min in one of the jugular venous lines followed by a maintenance infusion of 0.5 ml/h. The left ureter was catheterized with PE-10, and urine volume was determined gravimetrically. To determine glomerular filtration rate (GFR), a solution of normal saline containing [*methoxy*-^3H]inulin (10 μCi/ml) was infused at a rate of 1.2 ml/h after a priming dose of 0.5 ml over 5 min. A 23-gauge needle connected to a tuberculin syringe was inserted into the left renal vein to obtain renal venous samples. Filtration function was calculated from the extraction of inulin. Clearance data represented the mean of values obtained in two consecutive clearance periods. To infuse H_2O_2 into the renal circulation, a 34-gauge stainless steel Hamilton needle was inserted into the orifice of the left renal artery. The position of the tip of the needle was verified by an injection of lissamine green. GFR, renal plasma flow rates, mean arterial pressure, and urinary protein excretory rates were first determined in the basal state and for two consecutive 0.5-h periods after an intrarenal infusion of H_2O_2 in the left renal artery. 30 μmol of H_2O_2 was infused over 0.5 h. This model, first described by Yoshioka et al. (9) has been shown to induce markedly increased rates of urinary protein excretion. We examined the effect of acute elevation of plasma concentrations of pyruvate on H_2O_2-induced proteinuria. Before the infusion of H_2O_2, rats received a bolus of sodium pyruvate (1 ml/kg body weight of a 4% solution of sodium pyruvate in distilled water) given over 5 min followed by the same solution given as a maintenance infusion at 0.5 ml/h. Control rats received normal saline in identical volumes. In additional studies we tested the effect of another α-keto acid, 1.68% sodium α-ketoglutarate given at the same dose and rate as for sodium pyruvate. In other groups of rats we tested whether such acute administration of hydrogen peroxide led to increased kidney lipid peroxidation.

EFFECT OF PYRUVATE ON RENAL FUNCTION IN THE GLYCEROL MODEL OF ACUTE RENAL FAILURE

Functional studies 24 h after the administration of glycerol. We employed this model of acute renal injury because the kidney in this model produces increased amounts of hydrogen peroxide and exhibits

increased lipid peroxidation, whereas scavengers of free radicals ameliorate the structural and functional derangements in this model (3, 27, 28). To induce the model, male Sprague-Dawley rats in the weight range 250–300 g were deprived of drinking water but not rat laboratory diet (standard Purina rat Chow, Ralston Purina Co., St. Louis, MO) for 18 h before the administration of glycerol. A 50% glycerol solution at a dose of 7.5 ml/kg body weight was injected intramuscularly, half of the volume delivered into each thigh. To examine the protective effects of sodium pyruvate on renal function, we studied rats 24 h after the administration of glycerol to rats since at this time point, Guidet and Shah (3) have demonstrated that the kidney generates increased amounts of H_2O_2 in vivo. Rats were treated with either a solution of 8% sodium pyruvate in distilled water or equimolar glucose. Rats received either one of these treatments (2.5 ml/kg) by tail vein injection just before, and 8 and 16 h after the intramuscular injection of glycerol. 8 h after the last injection, renal function was determined by serum creatinine (measured by the Jaffe reaction on a Creatinine Analyzer II, Beckman Instruments, Inc.). The kidneys were then subjected to perfusion-fixation at the mean arterial pressure and morphometric analysis of kidney volume, intratubular cast volume, and volume of necrotic epithelium was performed by our previously described morphometric methods (29, and see below).

Morphometric studies 24 h after the administration of glycerol. We employed morphometric techniques to quantitate histologic injury rather than resorting to less precise qualitative or semiquantitative methods. We selected two histologic indices that can be readily and clearly identified: (a) intratubular casts which filled tubular profiles and (b) tubular epithelium exhibiting frank necrosis. We intentionally avoided scoring epithelium that exhibited sublethal injury since there is a recognized interobserver variability in assessing such injured epithelium. These histologic indices, intratubular casts, and tubular epithelial profiles exhibiting frank necrosis, are illustrated in Fig. 2. Morphometric analyses were performed in kidneys fixed by perfusion at mean arterial pressure of the rat with 1.25% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.4, osmolality 300 mosmol/kg) followed by immersion in fixative for 1 h. The perfused kidney was weighed, the length was measured with a vernier caliper to 0.1 mm (Vernier-type 6914, Science Ware, Pequanock, NJ), and the kidney was sliced into coronal sections using a stainless steel tissue slicer composed of razor blades aligned in a parallel fashion and spaced 2 mm apart. All slices except the terminal slice lacking the corresponding right-side cut surface were placed into cassettes with the right side up and embedded in paraffin, and sections were cut, 5 μm thick, and mounted onto a glass slide. Our morphometric analyses were performed in a blinded fashion and are described in detail in our previous publication (29).

Functional studies sequentially performed up to 3 h after the administration of glycerol. We also studied Sprague-Dawley rats with glycerol-induced acute renal injury for 3 h after the administration of glycerol. Rats were deprived of water but not rat chow for 18 h before study. On the morning of the study rats were set up for clearance studies as described above. In this study a bladder catheter, fashioned from PE-240 tubing was inserted into the bladder and thus GFR measurements were of both kidneys. Rats were treated with 8% sodium pyruvate, equimolar glucose, or equimolar sodium chloride given at a dose of 1 ml/kg as a bolus followed by 0.5 ml/h. 30 min after the initiation of the maintenance infusions, two clearance periods were undertaken for the determination of GFR. Glycerol (50% in distilled water) was then injected intramuscularly at a dose of 3.75 ml/kg and GFR was sequentially measured at hourly intervals for 3 h after the injection of glycerol. We also measured plasma creatine kinase as an index of muscle injury, 3 h after the administration of glycerol in rats treated with sodium pyruvate or equimolar sodium chloride or glucose. Creatine phosphokinase was determined by the creatine phosphokinase Sigma Kit (Sigma Chemical Co.). This is a colorimetric method that measures phosphorus generated by creatine phosphokinase in the presence of the substrates, creatine and ATP. In additional studies we measured lipid peroxidation in the kidney 3 h after the injection of glycerol in rats treated with sodium pyruvate or sodium chloride.

Statistical analysis

Values are presented as means±standard error of the means. Comparisons of two groups were performed with paired or unpaired Student's *t* test as appropriate. For experiments with three or more groups, one way ANOVA was performed with subsequent comparisons by Dunnett's test. Differences are considered significant for $P < 0.05$.

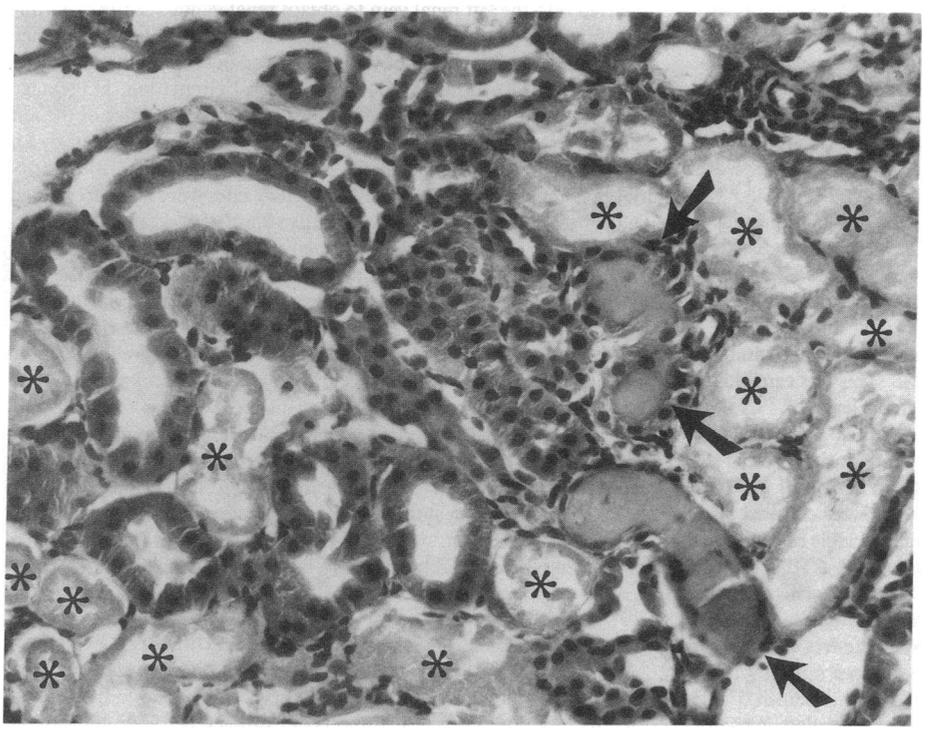

Figure 2. Histologic section of the kidney in a rat with glycerol-induced acute renal failure (×300). Morphometric analyses were performed for the determination of fractional and absolute fractional volumes of intratubular casts (*arrow*) and epithelial profiles with frank necrosis (*asterisks*).

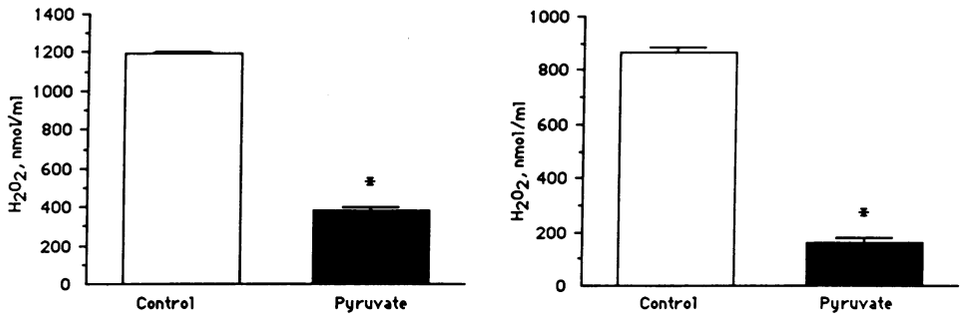

Figure 3. Scavenging of H_2O_2 by 1.5 mM pyruvate in the presence of 5 mM hypoxanthine and 40 mU xanthine oxidase (*left panel*), and 100 mg/dl glucose and 0.15 U/ml glucose oxidase (*right panel*). *$P < 0.001$ vs. pyruvate-free control. Values represent results of six separate determinations. Data: means±SE.

Results

Studies in vitro

Scavenging of H_2O_2 by pyruvate. H_2O_2 generated by enzyme systems closely paralleling those in the LLC-PK_1 monolayer studies was directly assayed in the absence and presence of 1.5 mM sodium pyruvate. H_2O_2 produced by hypoxanthine and xanthine oxidase was significantly reduced by pyruvate from 1,194.9±11.4 to 380.2±5.0 nmol/ml generated during 3 h of incubation (Fig. 3, *left panel*, $P < 0.001$). Similarly, pyruvate reduced H_2O_2 generated by glucose and glucose oxidase from 866.7±19.7 to 158.0±22.8 nmol/ml (Fig. 3, *right panel*, $P < 0.001$).

Effect of pyruvate on H_2O_2-induced lipid peroxidation. The stimulation of lipid peroxidation in kidney homogenate by 4 mM H_2O_2 as measured by content of TBARS was abolished in the presence of equimolar amounts of pyruvate. TBARS content of kidney homogenate treated with pyruvate (1.14±0.06 nmol/mg protein) was significantly less than TBARS content of kidneys treated with H_2O_2 and without pyruvate (1.56±0.11 nmol/mg protein, $P < 0.05$ by ANOVA), and not significantly different from the control incubations (1.04±0.13 nmol/mg protein, P = NS by ANOVA). The presence of 4 mM pyruvate did not affect the optical density readings obtained with the standard curve for malonaldehyde tetraethylacetal.

Effect of pyruvate on H_2O_2-induced injury to renal epithelial cells in culture. Fig. 4 demonstrates the cytoprotective effect of graded concentrations of pyruvate in the presence of a H_2O_2-generating system consisting of hypoxanthine and xanthine oxidase. Discernible at 0.250 mM, pyruvate significantly and in stepwise fashion prevented specific cytolytic ^{51}Cr release (Fig. 4, *left panel*). Values ranged from 57.0±3.9% in pyruvate free medium to 2.7±1.6% in the presence of 2 mM pyruvate ($P < 0.05$). Similarly, cell detachment was also diminished in the presence of pyruvate (Fig. 4, *right panel*).

Cytoprotection provided by 1.5 mM pyruvate in the presence of hypoxanthine and xanthine oxidase was compared with that provided by 1.5 mM malate, 100 μg/ml superoxide dismutase, and 100 μg/ml catalase (Fig. 5). Both catalase and pyruvate achieved marked and equivalent suppression of cytolytic ^{51}Cr release. Addition of malate was associated with increased cytolytic injury while superoxide dismutase had no effect (Fig. 5, *left panel*). Cell detachment followed a similar pattern with catalase and pyruvate significantly reducing detachment while malate and SOD had no demonstrable effect (Fig. 5, *right panel*). Studies utilizing glucose and glucose oxidase demonstrated similar findings (Fig. 6). Catalase and pyruvate markedly reduced both cell lysis and detachment. Malate provided no protection against peroxide-induced injury.

Sodium pyruvate (1.5 mM) also protected against cytolytic injury (40.5±1.7% vs. 68.0±0.9%, $P < 0.001$) and cell detachment injury (1.1±0.1% vs. 2.1±0.1%, $P < 0.001$) induced by equimolar amounts of reagent hydrogen peroxide. Sodium pyruvate did not protect against nonoxidant injury induced by 0.1% Triton X-100 (cell detachment injury 1.9±0.1% vs. 1.9±0.1%, P = NS; and cytolytic injury 78.0±1.0% vs. 79.0±1.0%, P = NS).

Quantitation of pyruvate decarboxylation induced by H_2O_2. Incubation of chromium loaded LLC-PK_1 cells in suspension with 1.5 mM H_2O_2 resulted in 41.1±0.9% specific ^{51}Cr release (Fig. 7, *left panel*). However, addition of 1.5 mM pyruvate to the incubation medium provided significant protection from H_2O_2-induced cytolysis resulting in 27.5±0.6% specific ^{51}Cr release ($P < 0.01$). Additionally, coincubation of [1-^{14}C]Na pyruvate with H_2O_2 and LLC-PK_1 generated 82.7±6.5 nmol/ml · h of CO_2. This greatly exceeded the amount of CO_2 produced in

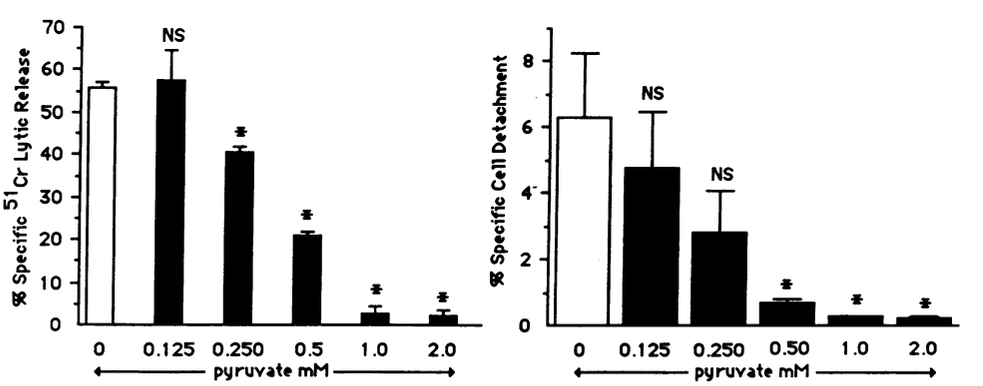

Figure 4. Effect of graded concentrations of pyruvate on LLC-PK_1 lytic injury (*left panel*) and cell detachment (*right panel*) induced by 5 mM hypoxanthine and 50 mU/ml xanthine oxidase. *$P < 0.05$ vs. pyruvate-free control. Values represent results of four experiments, each performed on quadruplicate wells. Data: means±SE.

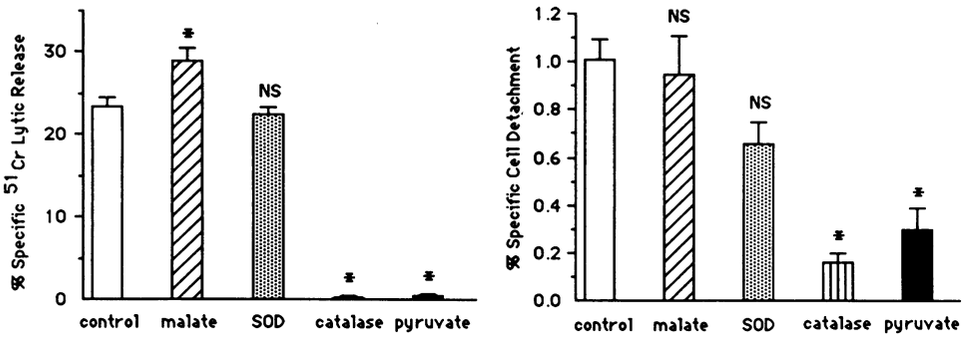

Figure 5. Comparison of cytoprotection provided by 1.5 mM pyruvate, 1.5 mM malate, 100 μg/ml SOD, and 100 μg/ml catalase in the presence of 5 mM hypoxanthine and 40 mU/ml xanthine oxidase as determined by cytolytic injury (*left panel*) and cell detachment (*right panel*). *$P < 0.05$ vs. control value. Values represent results of four experiments each performed on quadruplicate wells. Data: means±SE.

the control setting of LLC-PK_1 with [1-^{14}C]pyruvate alone (Fig. 7, *right panel*, $P < 0.001$). This latter finding agrees with previous observations demonstrating the glycolytic nature of LLC-PK_1 metabolism (30), and indicates that pyruvate does not constitute a major metabolic fuel in these studies.

Studies in vivo

EFFECT OF PYRUVATE ON H_2O_2-INDUCED PROTEINURIA

Body weight were identical in rats treated with sodium pyruvate or sodium chloride (388±4 vs. 388±4 g). In rats treated with sodium chloride, H_2O_2 induced markedly increased urinary protein excretory rates as compared to such excretory rates in the basal period, 8.2±2.3 vs. 139.6±23.7 μg/min, $P < 0.01$ (Fig. 8). This striking increment in protein excretion occurred in the absence of significant changes in mean arterial pressure (126±4 vs. 127±4 mmHg), in GFR of the left kidney (1.43±0.05 vs. 1.31±0.07 ml/min), or renal plasma flow rates (5.56±0.39 vs. 6.39±0.31 ml/min). Pretreatment with sodium pyruvate markedly attenuated the rise in urinary protein excretory rates induced by H_2O_2, 12.3±1.8 vs. 39.6±16.2 μg/min, P = NS, (Fig. 8) without any significant alteration in mean arterial pressure (125±8 vs. 124±8 mmHg), GFR of the left kidney (1.50±0.05 vs. 1.33±0.07 ml/min), or renal plasma flow rates (5.88±0.33 vs. 5.82±0.47 ml/min). Basal renal hemodynamics and mean arterial pressures in rats pretreated with pyruvate were not significantly different from control rats.

In additional studies, we infused 1.68% sodium α-ketoglutarate at the same dose and rate as studies utilizing 4% sodium pyruvate and 0.9% saline. In animals so treated hydrogen peroxide failed to evince significant increase in urinary protein excretory rates (8.2±1.7 vs. 50.7±36.3 μg/min, P = NS). Thus another α-keto acid, which like pyruvate, possesses the capacity to undergo nonenzymatic oxidative decarboxylation in the presence of hydrogen peroxide (18), protects against hydrogen peroxide-induced renal injury.

We also determined whether lipid peroxidation was increased in the kidney in rats subjected to an infusion of hydrogen peroxide in additional animals. Lipid peroxidation in the kidney subjected to hydrogen peroxide (0.80±0.1 nmol/mg protein, $n = 5$) was not significantly different from lipid peroxidation in the contralateral kidney not receiving hydrogen peroxide (0.93±0.07 nmol/mg protein, $n = 5$) or to the kidneys receiving N. saline alone (1.17±0.13 nmol/mg protein, $n = 4$). Thus although direct infusion of a reactive oxygen species, hydrogen peroxide, into the kidney evinces markedly increased rates of urinary protein excretion, these effects are not accompanied by increased lipid peroxidation in the kidney. These findings underscores the relative insensitivity of lipid peroxidation as a marker of oxidant stress.

EFFECT OF PYRUVATE ON RENAL FUNCTION AND STRUCTURE IN GLYCEROL-INDUCED ACUTE RENAL INJURY 24 h AFTER INJECTION OF GLYCEROL

Functional studies. Body weights in the rats treated with pyruvate or glucose just prior to the administration of glycerol were not different (276±3 vs. 279±3 g, P = NS) but were slightly less in the pyruvate treated animals 24 h later (250±3 vs. 261±3 g, $P < 0.05$). Serum creatinine measured 24 h after the induction of glycerol-induced acute renal failure was markedly lower in the animals treated with sodium pyruvate as compared to the animals treated with glucose, 0.9±0.1 vs. 1.4±0.1 mg/dl, $P < 0.02$. Thus, pyruvate as compared to equimolar amounts of glucose beneficially affects renal function as measured by serum creatinine in this in vivo model of increased H_2O_2 generation.

Structural studies. We also determined structural damage in glycerol-induced acute renal failure 24 h after the administration of glycerol in rats treated with pyruvate or glucose. We employed a quantitative method based on morphometric techniques rather than a qualitative or a semiquantitative one. Additionally, the histologic indices used to determine structural

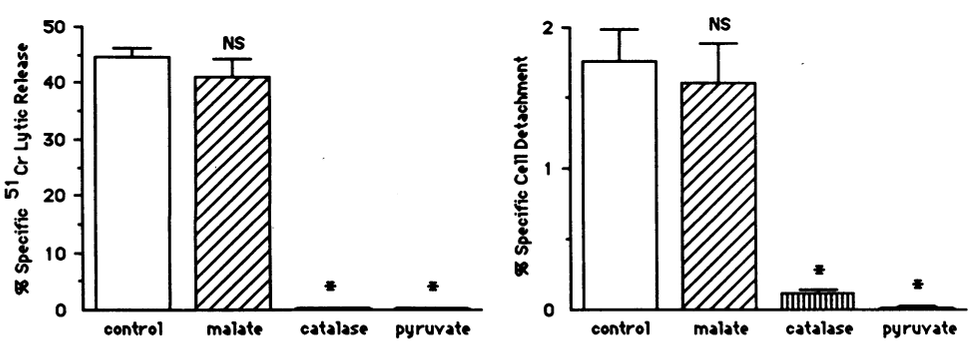

Figure 6. Comparison of cytoprotection by 1.5 mM pyruvate with 1.5 mM malate and 100 mg/ml catalase in the presence of 100 mg/dl glucose and 0.15 U/ml glucose oxidase as determined by cytolytic injury (*left panel*) cell detachment (*right panel*). *$P < 0.05$ vs. control. Values represent results of four experiments, each performed in quadruplicate. *$P < 0.05$ vs. control. Data: means±SE.

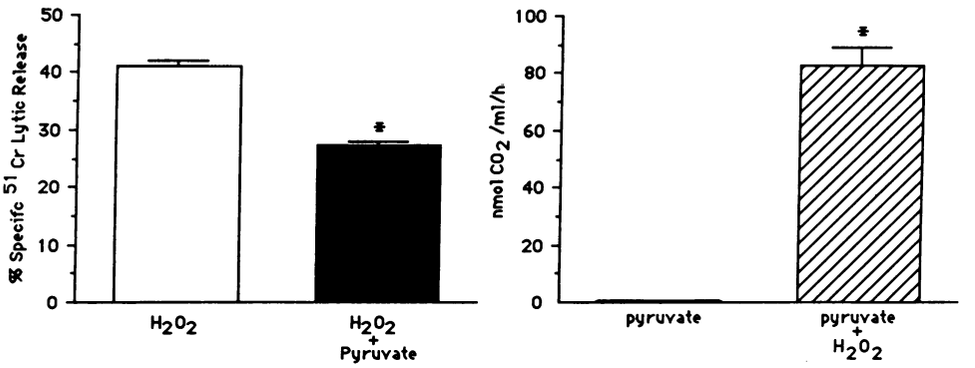

Figure 7. H_2O_2-induced cytolytic injury in the presence of equimolar pyruvate containing [1-^{14}C]pyruvate (*left panel*). Values represent results of six determinations. *$P < 0.01$ vs. pyruvate control. One-carbon decarboxylation of pyruvate in the presence and absence of 1.5 mM H_2O_2 from LLC-PK$_1$ culture wells (*right panel*). *$P < 0.01$. Values represent results of six determinations. Data: means±SE.

damage, that is, intratubular casts and tubular epithelium exhibiting frank necrosis, were clearly defined and free from the inherent subjectivity associated with scoring more subtle indices of renal damage. Kidney volumes were not significantly different in the two groups 525±22 vs. 563±30 mm^3, P = NS). However both the fractional volume as well as the absolute volume occupied by intratubular casts were markedly lower in animals treated with pyruvate as compared to animals treated with glucose (fractional volume 1.9±0.4 vs. 5.4±1.0%, $P < 0.005$; and absolute volume 9.9±2.3 vs. 29.8±4.2 mm^3, $P < 0.002$). Reduction in structural injury with pyruvate treatment was also seen with another index, that is, tubular epithelium with frank necrosis. Rats treated with pyruvate demonstrated marked reduction in the extent of epithelial necrosis measured both as fractional volume as well as absolute volume (fractional volume 6.5±1.2 vs. 27.3±1.4%, $P < 0.001$; and absolute volume 34.9±7.7 vs. 153.8±10.4 mm^3, $P < 0.001$). Thus, two morphometric indices demonstrated preservation of renal structure in animals treated with pyruvate. The beneficial of pyruvate in this model thus includes not only functional improvement but attenuation of structural injury.

Effect of pyruvate on renal function measured sequentially for 3 h after the injection of glycerol. We also studied rats at a relatively early stage after the injection of glycerol, that is up to 3 h after administration of glycerol. We compared animals pretreated with pyruvate to animals pretreated with glucose and animals pretreated with equimolar saline. We performed an additional control with hypertonic saline since some but not all studies have suggested that hypertonic saline may be beneficial in this model of acute renal injury (31, 32). Basal GFR in the three groups was not different. 1 h after the administration of glycerol there was a tendency for improved renal function as measured by GFR in the rats pretreated with pyruvate. 2 and 3 h after the administration of glycerol, GFR in the pyruvate-treated animals was markedly and significantly improved as compared to animals treated with either saline or glucose (Fig. 9). These studies demonstrate that pyruvate beneficially influences renal function at an early timepoint (1–3 h) as well at a delayed timepoint (24 h) after the administration of glycerol. To determine the whether pyruvate exerted protection at the level of muscle damage, we measured plasma creatine phosphokinase in rats treated with pyruvate or glucose. Creatine phosphokinase levels in rats treated with pyruvate was approximately 20% the value of rats treated with equimolar saline (9.5±1.0 vs. 51±10.2 Sigma Units, $P < 0.05$ by ANOVA) and 50% the value of rats treated with glucose (9.5±1.0 vs. 21.5±3.9, P = NS by ANOVA). Thus the beneficial effects exerted by pyruvate in glycerol-induced acute renal failure is derived, at least in part, by protective effects on muscle necrosis. In additional studies we measured lipid peroxidation in kidney tissue in rats 3 h after the intramuscular injection of 50% glycerol and treated with pyruvate or sodium chloride. Renal content of lipid peroxidation was not significantly different in the two groups (2.39±0.11 vs. 2.31±0.15 nmol/mg protein, P = NS).

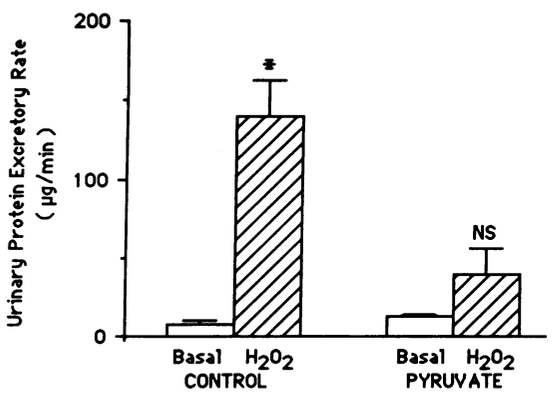

Figure 8. Effect of pyruvate on H_2O_2-induced urinary protein excretion. Control rats (n = 5) received N. Saline before and during the H_2O_2 infusion while the pyruvate-treated group (n = 6) received pyruvate before and during the H_2O_2 infusion. *$P < 0.01$ vs. basal protein excretion in controls and H_2O_2-induced protein excretion in pyruvate treated rats. Data: means±SE.

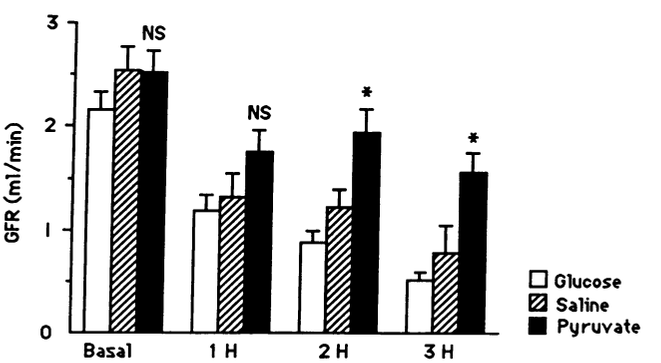

Figure 9. GFR measured sequentially in rats injected with 50% glycerol and treated with equimolar glucose (n = 9), saline, (n = 8) and pyruvate (n = 9). *$P < 0.05$, pyruvate vs. glucose and pyruvate vs. saline. Data: means±SE.

Discussion

Although recognized since 1904 as a scavenger for hydrogen peroxide (15), it was not until 1985 that the first evidence that pyruvate protected against H_2O_2-induced cellular injury in mammalian cells was provided by Andrae et al. (18). Similarly, pyruvate was shown to protect against H_2O_2-induced damage in murine malignant cellular lines (19) and transport activity in the lens (20). In the light of this capacity of pyruvate to scavenge H_2O_2 coupled to substantial evidence identifying H_2O_2 as a mediator of renal injury in disease models (1–10), we examined whether pyruvate affords protection against peroxide-induced injury in renal tissue in vivo and in vitro. We present multiple lines of evidence that, in their entirety, (*a*) confirm the potency of pyruvate in scavenging H_2O_2, (*b*) demonstrate that pyruvate protects against H_2O_2-induced injury in renal cells while concomitantly undergoing oxidative decarboxylation, and (*c*) attest to the remarkable effectiveness of pyruvate in protecting against H_2O_2-induced renal injury in vivo.

We utilized two models of H_2O_2-induced renal injury. We employed the elegantly simple model devised by Yoshioka et al. (9) in which the direct infusion of H_2O_2 into the renal artery provokes markedly increased rates of protein excretion. This model underscores the direct toxicity of H_2O_2 and adds further evidence that increased generation of H_2O_2 either by resident glomerular cells or invading inflammatory cells can disrupt glomerular permselective properties. In our studies we demonstrate that systemic infusion of pyruvate or another α-keto acid, α-ketoglutarate, which like pyruvate undergoes oxidative decarboxylation in the presence of hydrogen peroxide, attenuated this loss of glomerular permselectivity induced by H_2O_2. Thus, systemically administered pyruvate directly protects the kidney in vivo against damage confined to the kidney by intrarenal administration of H_2O_2. We suggest that oxidative decarboxylation of these α-keto acids underlie the attenuation of direct renal injury induced by hydrogen peroxide. However it is possible that other shared actions of α-keto acids may be involved.

Additional evidence for the protective effect of systemically administered pyruvate in models of H_2O_2-induced renal injury in vivo was provided by our studies undertaken in the glycerol model of acute renal failure. This model is an analogue of acute renal injury observed in the setting of rhabdomyolysis and tissue trauma. Guidet and Shah (3) have demonstrated markedly increased H_2O_2 production in this model. The administration of pyruvate over the 24-h period after the intramuscular injection of glycerol led to improvement in renal function. Such improvement in renal function was accompanied by marked reduction in structural injury as quantitated by our morphometric analyses of cast volume and the volume occupied by necrotic epithelium. We also studied rats with glycerol-induced acute renal injury pretreated with, and then subjected to sustained infusions of 8% sodium pyruvate, or equimolar glucose or equimolar sodium chloride for up to 3 h after the intramuscular injection of glycerol. Sequential measurements of GFR in rats so treated revealed approximately a twofold elevation in GFR in pyruvate-treated rats when compared to the rats treated with equimolar glucose or sodium chloride. Thus the protection afforded by the administration of pyruvate was observed both immediately as well at a relatively delayed time point after the injection of glycerol. The improvement in renal structure and function in this model observed with pyruvate was, at least in part, accounted for by reduction in muscle injury in rats treated with pyruvate. Interestingly, there is recent evidence that increased generation of hydrogen peroxide is involved in ischemic muscle injury (33). It is tempting to suggest that pyruvate protects at the muscle level by scavenging hydrogen peroxide generated in excess in postischemic muscle. However, despite roughly equivalent degrees of muscle injury as compared to glucose-treated rats, pyruvate-treated rats exhibited GFR that were 2.5-fold increased above glucose-treated rats. This suggests additional protective effect of pyruvate at the level of the kidney. It is also possible that other metabolic actions of pyruvate, apart from oxidative decarboxylation of hydrogen peroxide, may account for such protection extended to the kidney and muscle.

In the light of this property of pyruvate to scavenge H_2O_2, we offer two speculations. The first pertains to chronic renal injury. The decline of renal function in patients with chronic renal disease is retarded by a protein-restricted diet supplemented with α-keto acids (34, 35). The mechanism by which α-keto acids protect against chronic renal injury is unknown. Our recent studies undertaken in the remnant kidney model have demonstrated that increased dietary protein intake markedly increases the glutathione redox ratio (36). This ratio, the fraction of total kidney glutathione that exists in the oxidized form, provides an index of accumulation of H_2O_2 in the kidney. We speculate that a beneficial effect afforded by dietary supplementation with α-keto acids in the setting of chronic renal disease may derive, in part, from the scavenging of H_2O_2 by these acids.

We also suggest pyruvate is well suited to act as an intracellular scavenger for hydrogen peroxide for several reasons. The endpoint of the glycolytic pathway, pyruvate, is transported into the mitochondria via a carrier on the inner mitochondrial membrane (37). Once in the mitochondria, pyruvate undergoes oxidative decarboxylation to acetyl-coenzyme A and enters the tricarboxylic acid cycle (37). Pyruvate thus links glycolysis, a cytosol-residing pathway, to the tricarboxylic acid cycle which is housed in the mitochondrion and is the final common pathway for the oxidation of carbohydrates, lipids, and amino acids. H_2O_2 is generated at several cellular sites and is freely diffusible among cellular compartments (1, 2, 11). The availability of pyruvate in both cellular compartments thus provides a scavenger for hydrogen peroxide that has escaped detoxification by the glutathione peroxidase and catalase systems. The intracellular concentrations of pyruvate in the kidney are comparatively low ($\sim$ 30 μM) compared to plasma (50–100 μM) or to the liver (500 μM) (38, 39). The kidney generates large amounts of hydrogen peroxide in the course of oxidative metabolism (40, 41). These relatively low levels of pyruvate in the kidney may reflect the heightened rates of renal production of H_2O_2.

Our findings lead us to suggest that the administration of pyruvate offers a method of preventing or attenuating H_2O_2-induced injury in renal diseases. Millimolar concentrations of pyruvate in plasma, achieved by systemic administration of pyruvate, are tolerated in humans without any apparent adverse effects (42). Additionally, the ease with which pyruvate is transported into cells (43) and mitochondria (37) ensures that elevations in systemic concentrations of pyruvate are attended by increased pyruvate concentrations in the intracellular space in general, and in the mitochondrial compartment in particular. Administration of pyruvate may provide safe and effective

therapy for a variety of human renal diseases in which experimental analogues of these diseases clearly incriminate H_2O_2 in the pathogenesis of renal damage.

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