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Pyruvate Prevents Hydrogen Peroxide-Induced Apoptosis

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Studies were carried out to investigate the protective effects of pyruvate, a key glycolytic intermediate and α-keto-monocarboxylate, against oxidative stress-induced apoptosis. Oxidative stress was induced by treating mouse thymocytes with 25 μM hydrogen peroxide for 15 min at 37°C under 5% CO_2 in air. Pre- and post-treatment of cells with 10 mM pyruvate inhibited morphological changes, internucleosomal DNA fragmentation, and translocation of phosphatidylserine to the plasma membrane surface, which are characteristic features of apoptosis. L-lactate (10 mM) and acetate (10 mM) were ineffective in inhibiting apoptosis and appeared to be toxic to the cells under similar conditions. The results suggest that pyruvate has therapeutic potential for use in the treatment of oxidative stress-induced disorders associated with increased apoptosis.

Keywords: Apoptosis, oxidative stress, hydrogen peroxide, pyruvate, lymphocytes, flow cytometry

INTRODUCTION

Hydrogen peroxide has been implicated in a variety of oxidative stress-induced disorders including ischemia-reperfusion injury. Alzheimer's disease, Parkinson's disease, cerebellar degeneration, and radiation sickness.[1–5] These disorders are associated with excess cell loss. Hydrogen peroxide-induced oxidative damage to cellular membrane lipids,[6,7] proteins,[8,9] and DNA[10,11] results in cell death. Hydrogen peroxide inflicts cellular injury either directly or through free radicals generated by the Fenton reaction.[12] It can also kill cells by two mechanisms, necrosis and apoptosis. The severity of the damage to macromolecules determines which form of cell death will occur. Necrotic cell death results from acute cellular injury and is characterized by rapid cell swelling and lysis. Low concentrations of hydrogen peroxide induce apoptosis in a variety of cell types, while high doses induce necrosis.[13–16]

Apoptosis is a morphologically distinct form of cell death that is involved in many physiological and pathological processes. Apoptosis is characterized by cell shrinkage, cell surface blebbing,

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chromatin condensation, and DNA fragmentation into integer multiples of nucleosome or oligonucleosome sized units.[17] Membrane integrity is not affected in the initial stages of apoptosis, which is in marked contrast to what is seen in necrosis. However, alterations in the plasma membrane of apoptotic cells, such as translocation of phosphatidylserine to the outer surface, signal neighboring phagocytic cells to engulf them and thus complete the degradation process.[18,19] Cells not immediately phagocytosed break down into smaller membrane-bound structures called apoptotic bodies. The mechanism of oxidative stress-induced apoptosis involves activation of various signal transduction pathways, and the details of the process are far from clear.[20,21] The cell loss that occurs in neurodegenerative disorders appears to be primarily a result of oxidative stress-induced apoptosis.[22] In myocardial infarction and stroke, cells within the central area of ischemia appear to die rapidly as a result of necrosis. However, outside the central ischemic zone, cells die over a more protracted time period and, morphologically, appear to die by apoptosis.[23] Ischemia of both neurons and cardiac myocytes in culture results in the induction of apoptosis.[24,25] Reperfusion is associated with an acute burst in free radical production and cardiomyocytes die by apoptosis during reperfusion.[26] In addition, we have shown earlier that hydrogen peroxide produced during ionizing radiation kills lymphocytes by apoptosis.[27]

Hollemann first described in 1904 the capacity of pyruvate and related α-keto acids to reduce hydrogen peroxide to water while these acids concomitantly undergo non-enzymatic decarboxylation at the one-carbon position.[28] Subsequent studies demonstrated the protective role of pyruvate in hydrogen peroxide-induced renal injury, and ischemic and reperfusion injury *in vivo* and *in vitro*.[29–32] In the present study we investigated the protective effect of pyruvate vs acetate or lactate in inhibiting hydrogen peroxide-induced apoptosis using thymic lymphocytes as a model system. The results of our study indicated that 10 mM pyruvate inhibited the morphological and biochemical changes associated with peroxide-induced apoptosis. Acetate, a byproduct of pyruvate detoxification of H_2O_2, and lactate, produced by lactate dehydrogenase reduction of pyruvate, demonstrated no protective effect at 10 mM concentration and appeared to be toxic to the cells.

MATERIALS AND METHODS

Tissue Culture Medium

Tissue culture medium (TCM) consisted of RPMI 1640 medium supplemented with 25 mM HEPES buffer, 2 mM L-glutamine, 55 μM 2-mercaptoethanol, 100 U/ml pencillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B (all GIBCO/BRL, Grand Island, NY, USA), and 10% heat-inactivated fetal bovine serum (HyClone Laboratories, Logan, UT, USA).

Thymocyte Isolation

CD2F1 male mice, 6–7-weeks old were euthanized with CO_2. Thymuses were removed aseptically, rinsed in RPMI, and homogenized by pressing in a Stomacher device (Techman Co., Cincinnati, OH, USA) for 1 min. Single-cell suspensions were prepared by filtering the homogenate through 100 μm nylon mesh. Suspensions were washed once and resuspended in TCM. Cell numbers were obtained using a Coulter Counter (model ZM, Coulter Industries, Luton, Beds, England), and cell viability was estimated by the capacity of the cells to exclude trypan blue.

Hydrogen Peroxide Exposure and Pyruvate Treatment

Cells were resuspended in TCM at a density of 1×10^6 cells/ml. The cells were pre-incubated without and with different concentrations of pyruvate (0–20 mM) for 30 min at 37°C under

5% CO_2 in air. Cells were then exposed to 25 μM H_2O_2 for 15 min at 37°C under 5% CO_2 in air. Following 15 min incubation, catalase (100 U/ml) was added to all samples. The cells were pelleted by centrifugation (800*g* for 10 min) and resuspended in fresh TCM without and with the same concentrations of pyruvate (0–20 mM) and returned to the incubator.

DNA Fragmentation Assay

DNA fragmentation was assayed as previously described.[33] Briefly, cells (2×10^6) were collected by centrifugation (800*g* for 10 min), lysed with 0.2 ml of ice-cold lysis buffer (10 mM Tris-HCl, pH 7.5, 1 mM ethylenediaminetetraacetic acid, 0.2% Triton X-100), and centrifuged at 13,000*g* for 20 min to separate intact from fragmented DNA. The supernatant was conserved and the pellet resuspended in 0.2 ml of lysis buffer and sonicated for 10 s at 4°C. DNA concentration in the pellet and supernatant fraction was determined by an automated fluorometric method that we designed using AutoAnalyzer II components (Technicon, Tarrytown, NY, USA) and the DNA-specific fluorochrome Hoechst 33258 (Calbiochem-Behring, La Jolla, CA, USA). The percentage of DNA fragmentation refers to the ratio of the amount of DNA present in the 13,000*g* supernatant (fragmented) to the total of the DNA in the pellet (unfragmented) and 13,000*g* supernatant.

DNA Agarose Gel Electrophoresis

Electrophoresis of DNA was performed according to the method of Gong *et al.*,[34] which is particularly applicable to the qualitative detection of internucleosomal DNA fragments typical of apoptosis. Briefly, $1–2 \times 10^6$ cells were pelleted from the medium, washed once with Hank's balanced salt solution (HBSS), resuspended in 1 ml of HBSS, diluted with 10 ml of ice-cold 70% ethanol, and stored at −20°C for 24 h. The cells were then pelleted by centrifugation (800*g* for 10 min) and the ethanol completely removed. The pellet was resuspended and the cells lysed in 40 μl of phosphate–citrate buffer (192 parts of 0.2 M Na_2HPO_4 and 8 parts of 0.1 M citric acid, pH 7.8). After incubation at room temperature for 30 min, the cell lysate was centrifuged (1000*g* for 5 min) and the supernatant concentrated to about 20 μl using a Speed Vac concentrator (Savant Instruments, Farmingdale, NY, USA). A 3 μl aliquot of 0.25% Nonidet P-40 in distilled water was added to each sample followed by 3 μl of RNase A (1 mg/ml in water, Sigma Chemical Co., St. Louis, MO, USA) and the suspension incubated at 37°C for 30 min. A 3 μl aliquot of proteinase K (1 mg/ml in water, Boehringer Mannheim, Indianapolis, IN, USA) was added and the sample incubated for an additional 30 min at 37°C. Each sample was then mixed with an appropriate volume of 6× sample loading buffer (0.25% bromophenol blue/40% sucrose in water) and the entire mixture loaded onto a 0.8% agarose gel containing 0.5 μg ethidium bromide/ml. Electrophoresis was performed at 1.5 V/cm of gel length for about 16 h. DNA bands were visualized using UV transillumination, and photographs of gels were obtained using Polaroid Type 665 positive/negative film.

Microscopy

Cells ($0.5–1 \times 10^7$) were pelleted by centrifugation (800*g* for 10 min) and fixed by resuspension in 1 ml of freshly prepared 3% formaldehyde in ice-cold HBSS. Fixed cells could be maintained under refrigeration for several weeks without any apparent degradation. Cells were concentrated for microscopy by allowing them to settle by gravity to the bottom of a test tube at 4°C. Centrifugation to pellet cells was avoided because centrifugation of the fixed cells appeared to contribute to distorted morphology. For fluorescence microscopy, all but about 0.1 ml of the fixing buffer overlaying the cells was removed, and the cells were resuspended gently in the remaining buffer. A 20 μl aliquot was removed

and mixed with 20 µl of 0.1 mg/ml of ethidium bromide in HBSS (final concentration, 50 µg/ml). The stained suspension was kept in the dark on ice until used. Ten microliters of suspension was placed on a microscopic slide and gently covered with a 20-mm square cover slip. The cover slip was sealed with cement to prevent drying. Cells were allowed to settle and adhere to the surface of the slide for 5–10 min before beginning observation. Photomicroscopy was performed with an Olympus AHBT3 Research Microscope with Nomarski-type differential interference contrast and reflected-light fluorescence. Images were preserved on high-speed Polaroid Type 57 film.

Flow Cytometric Analysis

Cells were incubated with fluorescein isothiocyanate (FITC)-labeled annexin V and/or propidium iodide using an ApoAlert Annexin V Apoptosis kit (Clontech, Palo Alto, CA, USA). Briefly, 1×10^6 cells were washed with PBS and resuspended in 200 µl of 1× binding buffer (supplied in the assay kit). The cell suspension was incubated with annexin V-FITC (final concentration 1 µg/ml) and/or propidium iodide (final concentration 1 µg/ml) at room temperature for 5–15 min in the dark. The samples were analyzed by flow cytometry using a Becton Dickinson FACScan with Consort-32 operating system.

RESULTS

Cell death in thymic lymphocytes following exposure to 25 µM H_2O_2 for 15 min was characterized by morphological changes, DNA fragmentation, and plasma membrane changes. The effect of pyruvate on morphological changes in H_2O_2-treated thymic lymphocytes, fixed 24 h after exposure is shown in Figure 1. Panels A and B are bright-field and fluorescence photomicrographs of H_2O_2-treated cells. The cells exhibited morphological changes characteristic of apoptosis following exposure to 25 µM hydrogen peroxide. These morphological changes include cell shrinkage, chromatin condensation, membrane blebbing, nuclear fragmentation, and formation of apoptotic bodies. Some of the apoptotic bodies contain fragments of DNA. Cells treated with pyruvate before and after exposure to H_2O_2 exhibited morphology typical of normal cells with smoothly contoured cell membrane, uniform size, and similarly sized cells containing evenly distributed DNA (Panels C and D), indicating that pyruvate inhibits the morphological changes associated with oxidative stress-induced apoptosis.

The plasma membrane of a healthy cell generally exhibits an asymmetric distribution of its major phospholipids. Apoptosis is accompanied by a loss of membrane phospholipid asymmetry, resulting in the translocation of phosphatidylserine from the inner face of the plasma membrane to the cell surface.[35–38] Annexin V is a member of a family of proteins that has a strong affinity for phosphatidylserine.[38–41] In the present study, the changes in the phosphatidylserine distribution at the plasma membrane were assessed by flow cytometry following labeling of the cells with the fluorescein isothiocyanate conjugate of annexin V (FITC-annexin V). The effect of H_2O_2 and pyruvate on the translocation of phosphatidylserine to the outer surface of the plasma membrane in thymic lymphocytes is shown in Figure 2A–C. The results of dot-plot and histogram analysis are shown in Figure 2A and B, respectively. In both figures the cells on the right indicate annexin V positive cells. A small population of cells displayed a uniform binding to annexin V before ($5.6 \pm 0.7\%$; Figure 2C) and immediately after H_2O_2 exposure ($5.4 \pm 0.6\%$; Figure 2C). The population of cells labeled with annexin V increased to $92.2 \pm 1.4\%$ (Figure 2C) 24 h following exposure to H_2O_2. The number of annexin V positive cells increased to $38.4 \pm 1.2\%$ in cells not treated with H_2O_2 (Figure 2C), which suggests that a small population of control cells undergo apoptosis following incubation at 37°C under 5% CO_2 in air. H_2O_2 exposure increased

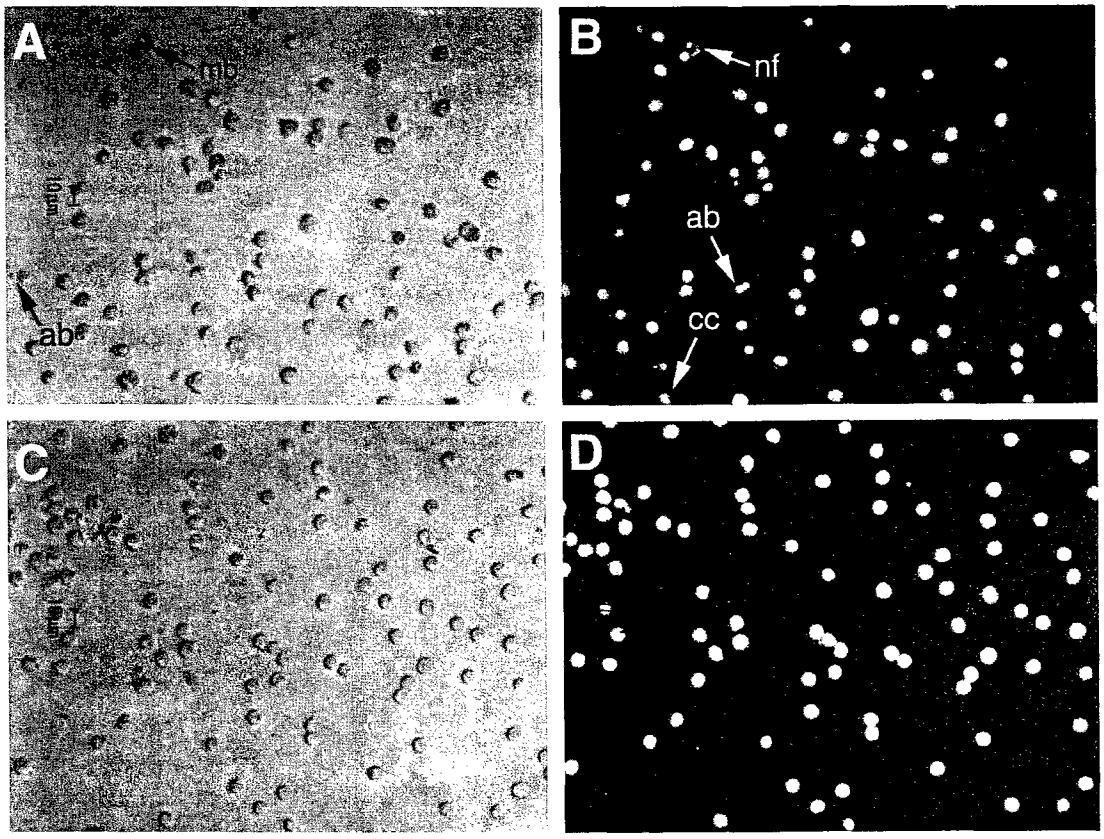

FIGURE 1 Effect of pyruvate on morphological changes in H_2O_2-treated thymic lymphocytes. Thymocytes were prepared for microscopy as described in Methods. Panels A and B show, respectively, bright-field and DNA-fluorescence images of H_2O_2-treated thymocytes 24 h after exposure to 25 μM H_2O_2 for 15 min. H_2O_2 induces morphological changes characteristic of apoptosis, including cell shrinkage, plasma membrane blebbing (mb), and formation of apoptotic bodies (ab). DNA staining shows pronounced chromatin condensation (cc), nuclear fragmentation (nf), and presence of DNA fragments in apoptotic bodies. Panels C and D demonstrate effect of pyruvate on morphology of H_2O_2-treated cells. Cells in these images were treated with 10 mM pyruvate before and after exposure to H_2O_2. Both bright-field and fluorescence images exhibit no apoptotic morphology.

the number of annexin V positive cells by 54%, compared to controls. When cells were incubated with pyruvate before and after exposure to H_2O_2 the number of annexin V positive cells decreased to that of cells not treated with H_2O_2 (36.8 ± 0.9%; Figure 2C), which suggests that pyruvate inhibits the H_2O_2-induced plasma membrane changes. Pyruvate did not reduce the number of annexin V positive cells (37.8 ± 0.9%; Figure 2C) that appeared in controls during *in vitro* incubation.

Figure 3 shows the effect of pyruvate on internucleosomal DNA fragmentation, one of the biochemical markers for apoptosis in thymic lymphocytes. The fragmented DNA isolated from H_2O_2-treated cells exhibited a ladder pattern of DNA fragments with size multiples of approximately 200 bp (lane 4). There was a small amount of DNA fragmentation detected in control cells (lane 2) that represented the background level of apoptosis. Pyruvate treatment reduced the level of DNA fragmentation in H_2O_2-treated cells to that of controls (lane 5), but it had no effect on the background level of DNA fragmentation that occurred in the controls (lane 3).

Quantitative measurements of DNA fragmentation were performed using a fluorometric

method, as described in Materials and Methods. The results shown in Table I indicate that the level of DNA fragmentation in H_2O_2-treated cells was reduced to that of control following pyruvate treatment, which suggests that pyruvate inhibits the H_2O_2-induced DNA fragmentation in thymic lymphocytes. The results also show that pyruvate had no inhibitory effect on the background DNA fragmentation that occurred in control cells. Acetate and lactate did not inhibit DNA fragmentation. In fact, they increased the level of DNA fragmentation in control cells. Acetate also increased the DNA fragmentation in H_2O_2-treated cells.

The results described so far indicated that pyruvate inhibits the H_2O_2-induced changes in morphology, translocation of phosphatidylserine to the plasma membrane surface, and internucleosomal DNA fragmentation in thymic lymphocytes. Further studies were carried out to investigate the protective effect of pyruvate on the survival of cells after H_2O_2 exposure. Cells were incubated with propidium iodide, which passes through the plasma membrane of dead cells and binds to DNA.[36] These dead cells can be enumerated by flow cytometry. The results shown in Figure 4A–C indicate that pyruvate protected the cells from H_2O_2-induced cell death.

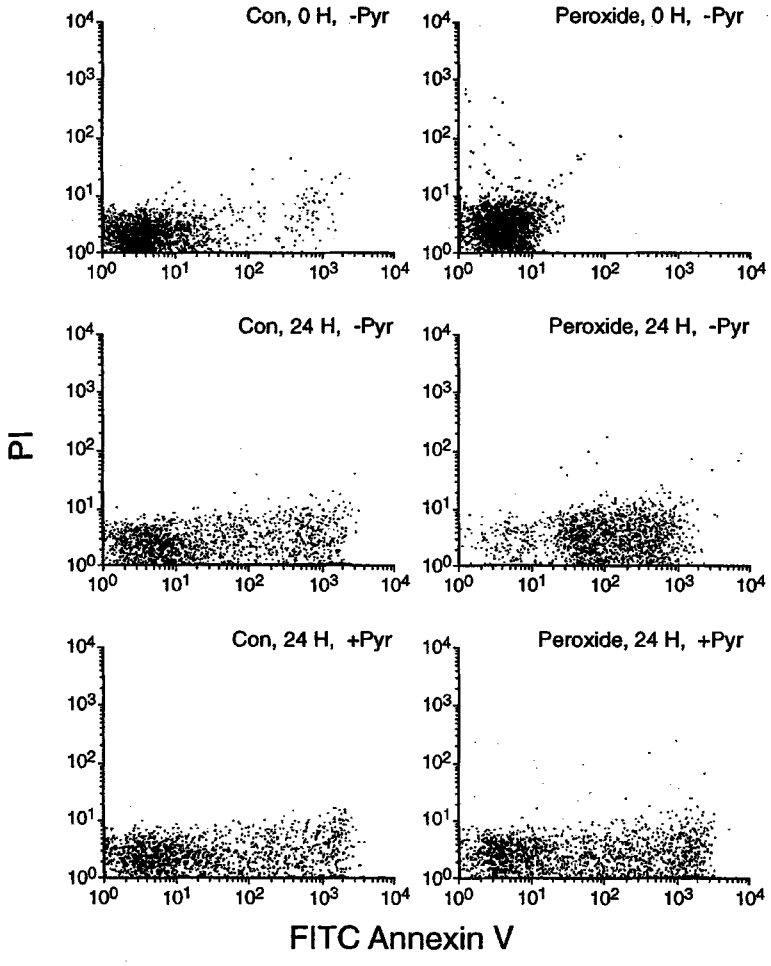

FIGURE 2A

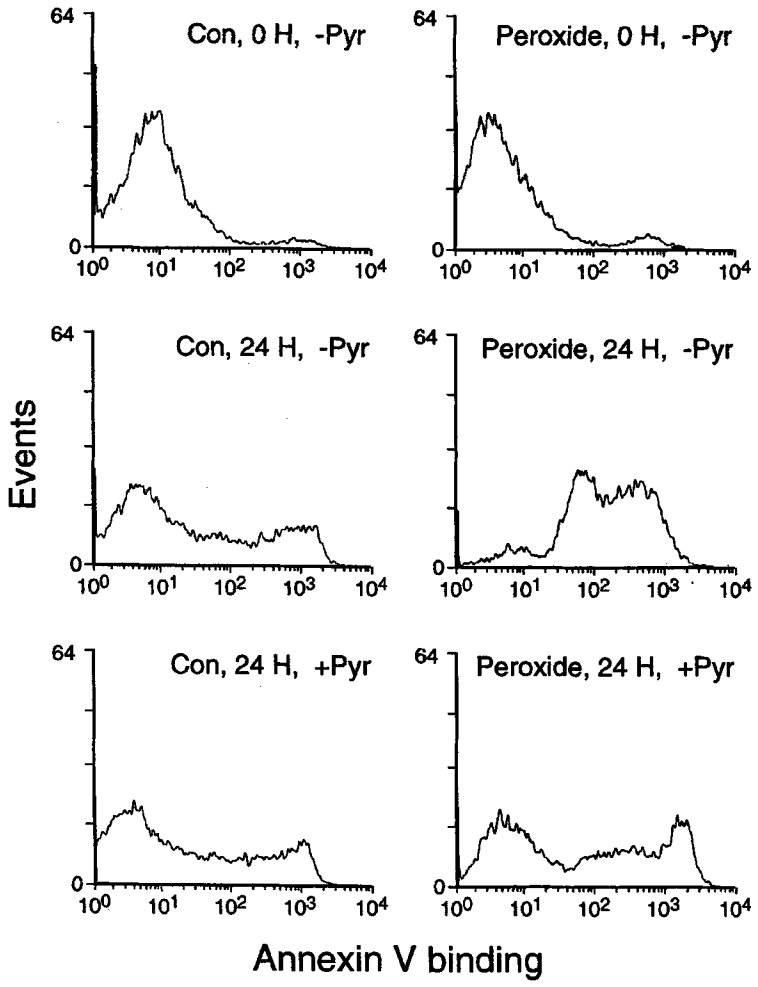

FIGURE 2B

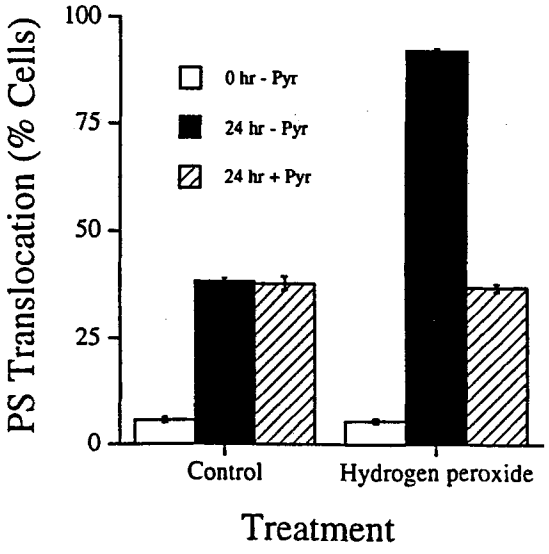

FIGURE 2C

FIGURE 2 Flow cytometric analysis of plasma membrane changes associated with H_2O_2-induced apoptosis. Thymocytes (1×10^6 cells) were labeled with FITC-annexin V using ApoAlert Annexin V Apoptosis kit. The samples were analyzed by flow cytometry using a Becton Dickinson FACScan with Consort-32 operating system. In each sample 10,000 cells were analyzed. A: Dot-plot analysis. The cells towards the right indicate annexin V positive cells. B: Histogram analysis. Annexin V positive cells appear towards the right. C: Quantitative analysis of annexin V binding. Con −Pyr, control without H_2O_2 without pyruvate; Con +Pyr, control without H_2O_2 with pyruvate. The concentration of pyruvate was 10 mM. The results are mean ± SEM of three independent experiments.

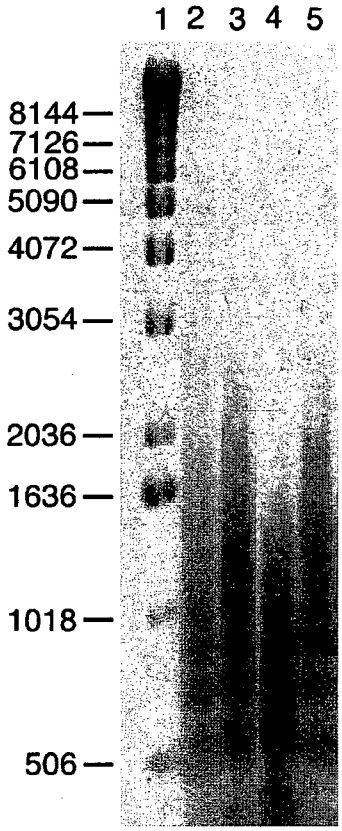

FIGURE 3 Effect of pyruvate on internucleosomal DNA fragmentation. Fragmented DNA was extracted from cells after various treatments and electrophoresed as described in Methods. Lane 1, standard 1 kb DNA ladder; lane 2, DNA from control cells; lane 3, DNA from control cells incubated with 10 mM pyruvate; lane 4, DNA from H_2O_2-treated cells; lane 5, DNA from H_2O_2-treated cells after incubation with pyruvate.

TABLE I Effect of pyruvate on internucleosomal DNA fragmentation: quantitative analysis

Condition	DNA fragmentation (%)		*p*
	Control	25 μM H_2O_2	
No pyruvate	29.21 ± 2.38	42.02 ± 1.01	< 0.05
5 mM pyruvate	27.95 ± 1.71	33.17 ± 2.38	NS
10 mM pyruvate	28.67 ± 1.93	31.27 ± 2.02	NS
20 mM pyruvate	29.50 ± 1.98	30.92 ± 2.19	NS
10 mM acetate	36.26 ± 2.27	46.79 ± 1.83	< 0.05
10 mM L-lactate	39.30 ± 1.20	41.04 ± 1.20	NS

Thymocytes were incubated with pyruvate, acetate, L-lactate before and after exposure to H_2O_2. Fragmented DNA was separated from intact DNA and analyzed as described in Methods. The results are mean ± SEM of 12 measurements from four independent experiments.

The propidium iodide positive (dead) cells appear towards the upper left in the dot-plot analysis (Figure 4A) and towards the right in the histogram analysis (Figure 4B). The number of dead cells increased from 33.6 ± 1.4% (control 24 h; Figure 4C) to 72.2 ± 1.4% (Figure 4C) 24 h following a 15 min exposure to 25 μM H_2O_2. Following pyruvate treatment the number of dead cells in the H_2O_2-exposed population decreased to 32.9 ± 1.2% (Figure 4C). Again, pyruvate did not protect the control cells (32.7 ± 1.2%), which die by apoptosis during the 24 h *in vitro* incubation.

DISCUSSION

The results of the present study indicate that thymic lymphocytes die by apoptosis following exposure to 25 μM H_2O_2 for 15 min. Our earlier studies showed that exposure to 0.5–10 μM H_2O_2 for 10 min also induced apoptosis in these cells.[15] We observed that exposure to higher concentrations of H_2O_2 (> 50 μM) induced necrosis (results not shown) in these cells. The cells exhibited characteristics of both apoptosis and necrosis, including morphological changes, and random DNA cleavage along with internucleosomal breaks, following prolonged incubation (> 30 min) with 25 μM H_2O_2 (results not shown). This suggests that these cells undergo secondary necrosis after prolonged exposure to H_2O_2. In the present study, the secondary necrosis was prevented by the addition of catalase after the 15 min H_2O_2 exposure.

The results clearly indicate that pyruvate protects thymic lymphocytes from H_2O_2-induced apoptotic death. Pyruvate inhibited the morphological changes, translocation of phosphatidylserine to the plasma membrane surface, and internucleosomal fragmentation of DNA in cells exposed to 25 μM H_2O_2 for 15 min. However, pyruvate did not reduce the number of annexin V positive cells that appeared in controls during *in vitro* incubation (Figure 2). In addition, there

is a background level of DNA fragmentation (29.21 ± 2.38%) in controls following 24 h incubation that could not be inhibited by pyruvate (Table I). The results indicate that pyruvate could not protect approximately 30% of the control cells which die by apoptosis after *in vitro* incubation. It is possible that these control cells might have been committed to die by apoptosis before the addition of pyruvate. Apoptosis involves activation of several signal transduction pathways that are cell and stimuli specific. It is also possible that the signal transduction pathway by which the control cells die during *in vitro* incubation may be different from that activated by H_2O_2. Pyruvate may not be able to inhibit the pathway by which the control cells die during *in vitro* incubation. It has been suggested by Sellins and Cohen[42] that the background level of apoptosis seen in thymocytes during *in vitro* incubation is related to suboptimal culture conditions and pre-existing (*in vivo*) influences, e.g., exposure to glucocorticoids.

The present studies also indicate that pyruvate is effective in protecting the thymic lymphocytes from oxidative stress-induced apoptosis at 5–20 mM concentration (Table I). Pyruvate concentrations less than 5 mM were not effective in inhibiting apoptotic death (results not shown).

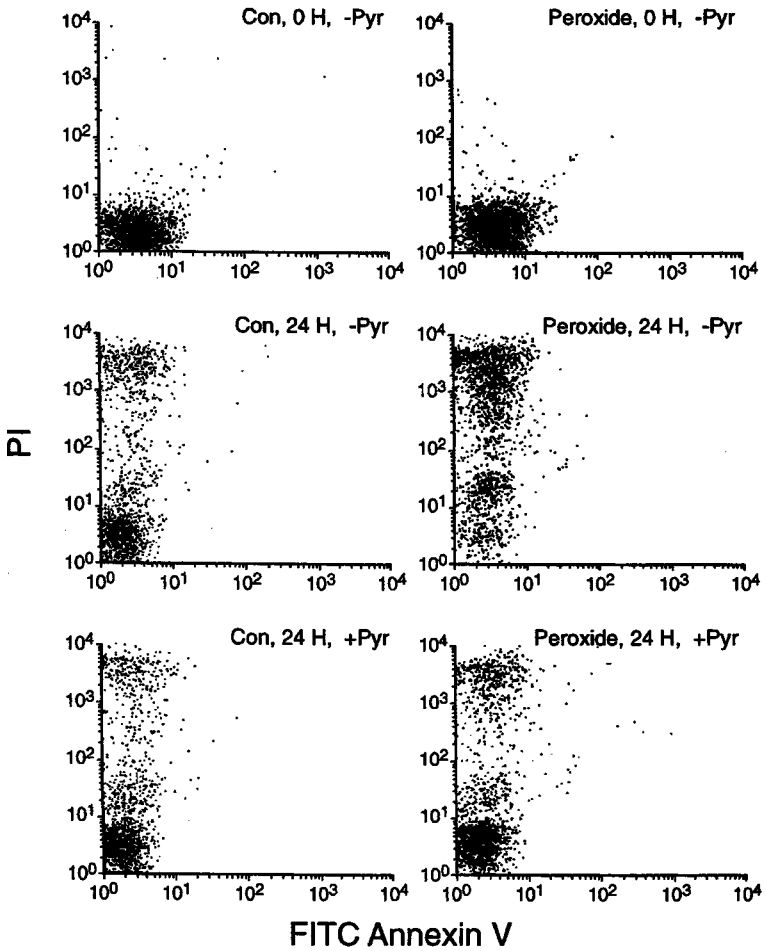

FIGURE 4A

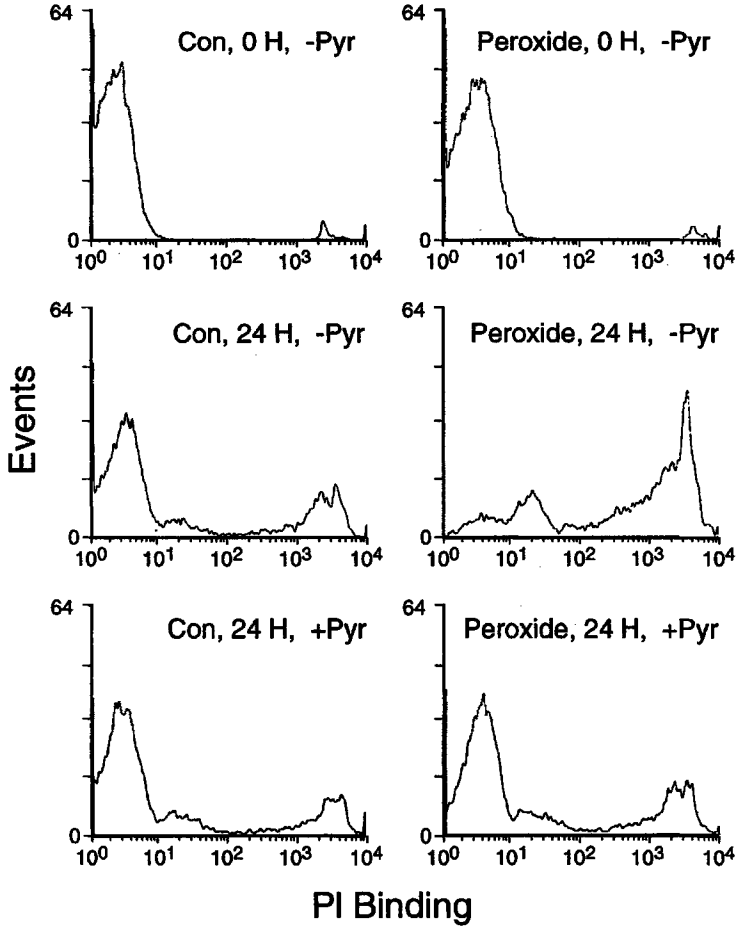

FIGURE 4B

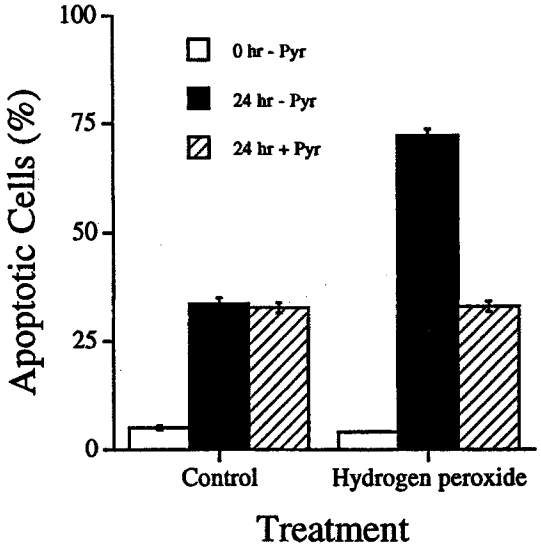

FIGURE 4C

FIGURE 4 Effect of pyruvate on cell viability. Thymocytes were labeled with propidium iodide after various treatments and analyzed by flow cytometry. In each sample 10,000 cells were analyzed. A: Dot-plot analysis. The cells towards the upper left are propidium iodide positive (dead) cells. B: Histogram analysis. Propidium iodide positive cells appear towards the right. C: Quantitative analysis of propidium iodide positive (dead) cells. Con −Pyr, control without H_2O_2 without pyruvate; Con +Pyr, control without H_2O_2 with pyruvate. The concentration of pyruvate was 10 mM. The results are mean ± SEM of three experiments.

It is possible that pyruvate may be inhibiting the apoptotic process by more than one mechanism. Apoptosis is associated with or preceded by substantial cytosolic deenergizations (large decrease in cytosolic $[ATP]/[ADP] * [P_i]$) due to mitochondrial failure or permeability transitions and inner membrane depolarizations.[43–45] This will inhibit the energy-linked Na^+ and Ca^{2+} pumps vital for intracellular calcium homeostasis; such conditions can result in sustained elevations of intracellular Ca^{2+} and trigger apoptosis.[43] Pyruvate, at 5–10 mM concentrations, elevates cytosolic $[ATP]/[ADP] * [P_i]$ and maintains the intracellular calcium homeostasis.[30,46,47] This may be one of the mechanisms by which pyruvate protects cells from apoptosis. Equimolar concentrations of acetate or lactate (5–10 mM) do not increase $[ATP]/[ADP] * [P_i]$.[30,47] Also, in cell-free systems, pyruvate can protect the activity of SH-dependent enzymes, such as cardiac and renal Na^+-, K^+-ATPases, against oxidant injury.[32,48] A redox imbalance caused by a decrease in antioxidants seems to play a role in apoptosis.[20,21,49] The antiapoptotic concentrations of pyruvate (5–10 mM) double the $NADPH/NADP^+$ and GSH/GSSG ratios and enhance the reductive potentials of the glutathione redox system.[30,46,47] In addition, pyruvate is a potent non-enzymatic scavenger of H_2O_2. It detoxifies H_2O_2 on a 1 : 1 molar basis, by an interaction between its carbonyl group and H_2O_2 to yield acetate and CO_2.[50,51] Acetate and lactate do not scavenge H_2O_2.[51,52] Exogenous catalase, too, is highly effective in detoxifying H_2O_2, but it is not permeable to intact cell membranes, and therefore removes H_2O_2 in the extracellular space only.[53] In contrast, exogenous pyruvate has monocarboxylate-carrier facilitated access to the cytosol, nucleoplasm, and mitochondrial matrix,[54] and may be more effective than exogenous catalase in preventing oxidant injury intracellularly. Studies indicate that endogenous pyruvate scavenges H_2O_2 in biofluids, including human serum and inflamed knee-joint synovial fluid.[52]

Results shown in Table I indicate that acetate and lactate increased the level of DNA fragmentation in controls and acetate alone increased the DNA fragmentation in H_2O_2-treated cells. The concentrations of acetate and lactate used in the present study (10 mM) appear to be toxic to thymic lymphocytes. High concentrations of lactate (5–10 mM) are known to increase the intracellular H^+ ion concentration, which can induce cytotoxicity.[30,47] High concentrations of acetate (5–10 mM) lower $[ATP]/[ADP] * [P_i]$,[55] which can induce apoptosis.[43–45] At lower physiological concentrations (0.5–1 mM), these compounds, though non-toxic, were not effective in inhibiting apoptosis (results not shown). The results shown in the present and previous [30,46,47] studies indicate that 10 mM pyruvate is not toxic to the cells. The amount of acetate formed due to direct interaction of pyruvate with 25 μM H_2O_2 will be too low to be toxic because pyruvate and H_2O_2 interact on a 1 : 1 molar basis. Millimolar concentrations of pyruvate in plasma, achieved by systemic administration of pyruvate, are tolerated in humans without any apparent adverse effects.[56,57]

Increased cell loss through apoptosis has been reported in several oxidative stress-induced pathological conditions, including reperfusion injury of ischemic tissues, renal injury, oxygen toxicity of the lung, Parkinson's disease, the familial form of amyotrophic lateral sclerosis, Alzheimer's disease, cerebellar degeneration, stroke, aplastic anemia, and AIDS.[1–4,22] Pyruvate has been shown to protect the heart and kidney from reperfusion injury *in vivo* and *in vitro*.[29–32] The results of the present study suggest that pyruvate has the therapeutic potential for use in the treatment of oxidative stress-induced disorders associated with increased apoptosis.

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