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Pyruvate Protects Mitochondria from Oxidative Stress in Human Neuroblastoma SK-N-SH Cells

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Abstract

Oxidative stress is implicated in neurodegenerative diseases including stroke, Alzheimer's disease and Parkinson's disease, and has been extensively studied as a potential target for therapeutic intervention. Pyruvate, a natural metabolic intermediate and energy substrate, exerts antioxidant effects in brain and other tissues susceptible to oxidative stress. We tested the protective effects of pyruvate on hydrogen peroxide (H₂O₂) toxicity in human neuroblastoma SK-N-SH cells and the mechanisms underlying its protection. Hydrogen peroxide insult resulted in 85% cell death, but co-treatment with pyruvate dose-dependently attenuated cell death. At concentrations of ≥ 1 mM, pyruvate totally blocked the cytotoxic effects of H₂O₂. Pyruvate exerted its protective effects even when its administration was delayed up to 2 hr after H₂O₂ insult. As a scavenger of reactive oxygen species (ROS), pyruvate dose-dependently attenuated H₂O₂-induced ROS formation, assessed from 2,7-dichlorofluorescein diacetate fluorescence. Furthermore, pyruvate suppressed superoxide production by submitochondrial particles, and attenuated oxidative stress-induced collapse of the mitochondrial membrane potential. Collectively, these results suggest pyruvate protects neuronal cells through its antioxidant actions on mitochondria.

Keywords

pyruvate; mitochondria; oxidative stress; neuroprotection; hydrogen peroxide; superoxide

1. INTRODUCTION

The central nervous system is particularly vulnerable to oxidative damage due to its high energy expenditure and oxygen demand. Elevated concentrations of free radicals and resultant oxidative damage, such as lipid peroxidation and protein carbonylation, have been repeatedly demonstrated in neurodegenerative disorders, such as Alzheimer's disease, Parkinson's disease and ischemic stroke [2]. Accordingly, antioxidants have been extensively studied as potential therapies for various neurodegenerative diseases.

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Mitochondria, which generate ATP by oxidative phosphorylation to meet the brain's energy demands, rank among the principal intracellular targets of reactive oxygen species (ROS). These unstable compounds inflict oxidative damage on cellular proteins, lipids, and nucleic acids. Oxidative damage to mitochondrial membranes, enzymes and respiratory chain components culminate in the impairment of mitochondrial ATP production. Moreover, ROS are known to interact with physiologic signaling mechanisms and can initiate apoptotic or necrotic cell death. Indeed, mitochondria dysfunction is associated with various neurodegenerative diseases and brain ischemic injury [6,12,16,17].

An aliphatic monocarboxylate and glycolytic end product, pyruvate enters mitochondria via the inner membrane monocarboxylate transporter and assumes a central role in cellular energy production. Moreover, pyruvate embodies antioxidant properties due to its α -ketocarboxylate structure, enabling it to directly neutralize peroxides and peroxynitrite, and its mitochondrial metabolism, which generates NADPH to maintain glutathione reducing power [31]. Pyruvate has been shown to be neuroprotective in various *in vitro* and *in vivo* models of oxidative stress. In neuronal cell cultures, pyruvate protects against various insults such as β -amyloid [1], H_2O_2 [13,26,53], mitochondrial toxins [34] and zinc [11,27]. In *in vivo* models, pyruvate and its derivatives have been shown to protect against cerebral ischemic injury [28,36,55] and zinc toxicity [28].

This study tested the effects of pyruvate against oxidative stress in human neuroblastoma SK-N-SH cells, and sought to define the role of mitochondria in mediating cytoprotection by pyruvate. We demonstrated that pyruvate remarkably abrogated H_2O_2 toxicity in SK-N-SH cells by its direct antioxidant protection of mitochondria.

2. RESULTS

2.1. Cytoprotection by pyruvate against oxidative stress

Exposure of SK-N-SH cells to 150 μM H_2O_2 for 18 hr resulted in 85% cell death as measured by calcein AM assay (Figure 1A). Sodium pyruvate co-treatment dose-dependently increased cell survival at concentrations from 100 μM to 4 mM. At concentrations ≥ 1 mM, pyruvate essentially prevented H_2O_2 -induced cell death (Figure 1A).

Additional experiments tested whether pyruvate could protect cells even when its administration was delayed up to 2 hr after H_2O_2 exposure. Administration of sodium pyruvate at 30 min (Figure 1B) or 1 hr (Figure 1C) after H_2O_2 provided significant protection against the oxidative insult. When given 1 hr after H_2O_2 , 100 μM and 1 mM sodium pyruvate increased cell survival from $16 \pm 2\%$ to $55 \pm 3\%$ and $79 \pm 3\%$, respectively. Even when administered 2 hr after H_2O_2 , pyruvate provided significant protection against H_2O_2 -induced cytotoxicity, albeit with decreased efficacy (Figure 1D).

The cytoprotective effects of pyruvate were also examined by MTT assay (Figure 2). Exposure of cells to 150 μM H_2O_2 for 18 hr decreased MTT reduction to $37 \pm 1\%$ of baseline. Pyruvate co-treatment dose-dependently mitigated the loss of MTT reduction caused by H_2O_2 : 0.1 mM pyruvate increased MTT reduction to $49 \pm 3\%$ of baseline, and 1–4 mM pyruvate nearly restored the activity to c. 90% of baseline (Figure 2).

2.2. Effect of pyruvate on hydrogen peroxide-induced free radical generation

The antioxidant effects of pyruvate on intracellular and mitochondrial reactive oxygen species (ROS) were respectively examined by monitoring DCFH-DA and DHR fluorescence. DCFH-DA fluorescence revealed a progressive increase in intracellular ROS to $609 \pm 12\%$ of H_2O_2 -free control following 1 hr exposure to 150 μM H_2O_2 (Figure 3A). Co-treatment with 1 and 2 mM sodium pyruvate sharply attenuated intracellular ROS accumulation to 220 ± 5 and 126

$\pm 5\%$ of control, respectively (Figure 3A). DHR fluorescence, a measure of mitochondrial ROS, revealed a similar pattern of responses to H_2O_2 and sodium pyruvate (Figure 3B). H_2O_2 alone increased DHR fluorescence up to 10-fold within 30 min. Pyruvate dose-dependently attenuated mitochondrial ROS generation (Figure 3B), although to a somewhat lesser extent than its suppression of intracellular ROS (Figure 3A).

2.3. Effect of pyruvate on superoxide production by submitochondrial particles

Inhibition of mitochondrial respiratory complexes can increase $\cdot\text{O}_2^-$ production. We assessed effects of pyruvate on $\cdot\text{O}_2^-$ production by SMP under three conditions: no mitochondrial complex inhibitor; mitochondrial complex I inhibitor, rotenone; mitochondrial complex III inhibitor, antimycin A. In the absence of respiratory complex inhibitors, $\cdot\text{O}_2^-$ generation rate was $0.79 \pm 0.09 \text{ pmol} \cdot \text{mg protein}^{-1} \cdot \text{min}^{-1}$; treatment with 0.1 and 1 mM pyruvate lowered the rate to 0.38 ± 0.10 and $0.50 \pm 0.07 \text{ pmol} \cdot \text{mg protein}^{-1} \cdot \text{min}^{-1}$, respectively (Figure 4A). In the presence of respiratory complex inhibitors, rotenone (Figure 4B) and antimycin A (Figure 4C), $\cdot\text{O}_2^-$ production increased to 1.16 ± 0.12 and $1.31 \pm 0.06 \text{ pmol} \cdot \text{mg protein}^{-1} \cdot \text{min}^{-1}$, respectively. Pyruvate at 0.1 and 1 mM markedly reduced $\cdot\text{O}_2^-$ generation in the presence of the respiratory inhibitors (Figure 4B, C).

2.4. Effect of pyruvate on mitochondrial membrane potential

In the FRET assay, 30 min exposure to 3.0 mM H_2O_2 caused $\Delta\Psi_m$ collapse in SK-N-SH cells. As expected, the concentration of H_2O_2 required to cause acute collapse of $\Delta\Psi_m$ (*i.e.*, 3 mM) was substantially more than that required for long term cytotoxicity (*i.e.*, 0.15 mM). H_2O_2 rapidly induced $\Delta\Psi_m$ collapse, evidenced by the increase of NAO fluorescence due to efflux of TMR from the mitochondrial matrix. Pyruvate concentration-dependently reduced the magnitude of mitochondrial depolarization induced by H_2O_2 (Figure 5A).

Pyruvate protection of $\Delta\Psi_m$ during H_2O_2 exposure was confirmed by confocal microscopy, using JC1 as $\Delta\Psi_m$ indicator (Figure 5B). H_2O_2 insult induced $\Delta\Psi_m$ collapse, indicated by the progressive loss of red J-aggregate fluorescence and increase of green monomer fluorescence, producing a shift from red to yellow in the confocal images (Figure 5B). The $\Delta\Psi_m$ collapse induced by H_2O_2 was suppressed by 2 mM pyruvate.

3. DISCUSSION

Two important findings resulted from this study. First, pyruvate exerts significant protection of neuronally derived SK-N-SH cells against H_2O_2 -induced oxidative stress in both co-treatment as well as delayed treatment protocols. Concordant with reports of other investigators, the cytoprotective effects of pyruvate are concentration-dependent, and are afforded by physiological (high μM) to pharmacological (low mM) concentrations [13,26]. The therapeutic window of pyruvate administration extends up to 2 hr after H_2O_2 insult, suggesting pyruvate protects cells by mechanisms other than its direct nonenzymatic reaction with H_2O_2 . Second, pyruvate cytoprotection against oxidative insult is mediated through its direct action in mitochondria, as evidenced by its dampening of mitochondrial ROS generation and its stabilization of mitochondrial membrane potential maintenance under oxidative stress.

The SK-N-SH neuroblastoma cell line was developed by Biedler *et al.* [7], and is used extensively as a target cell line in cytotoxicity assays. SK-N-SH cells exhibit a neuronal phenotype with expression of multiple neurochemical markers [8]. SK-N-SH cells respond to numerous insults including β -amyloid, mitochondrial permeability transition, and serum deprivation, indicating that this cell line could be very useful in the assessment of neurotoxicity and neuroprotection [4]. SK-N-SH cells have been used by this and other laboratories [5,20, 21,46,48–51,56] as an *in vitro* model for studying potential neuroprotection mechanisms.

Peroxides are generated continuously by cells that consume oxygen. Among the different peroxides, H_2O_2 is the one formed in the highest quantities [15]. H_2O_2 is a major reactive oxygen intermediate and a by-product of normal cellular metabolism produced by superoxide dismutase (SOD) and monoamine oxidase (MAO). H_2O_2 can easily penetrate cellular membranes and exert its toxic effects through either the ferrous iron-dependent or $\cdot O_2^-$ -driven formation of the highly reactive hydroxyl radical ($\cdot OH$), which attacks and modifies lipids, proteins and DNA, or by altering cytosolic redox status [23]. Normally, cellular concentrations of H_2O_2 and redox-active iron are kept low by efficient disposal of H_2O_2 and by storage of iron in ferritin, respectively. However, in pathological situations including various neurodegenerative diseases, the detoxification of H_2O_2 is compromised in neurons and other cell types, and H_2O_2 accumulates and triggers cell death cascades [15,24]. High concentrations of H_2O_2 have been detected in the brain after ischemia-reperfusion injury [40] [25]. In striatum, H_2O_2 concentrations could exceed 100 μM after ischemia-reperfusion insult [25]. Such concentrations of H_2O_2 have been shown to be toxic to PC12 cells and rat primary hippocampal neurons [25]. In this study, 18 hr exposure to 150 μM H_2O_2 caused 85% death of SK-N-SH cells. Consistent with reports from other laboratories [13,26,53], pyruvate dose-dependently prevented H_2O_2 -induced cell death. Moreover, this study demonstrated that pyruvate afforded significant neuroprotection even when its administration was delayed up to 2 hr after oxidative insult.

Multiple mechanisms have been suggested to contribute to the cytoprotective effect of pyruvate. Pyruvate enters cells by a specific H^+ -monocarboxylate cotransporter [18,41]. A readily oxidized metabolic fuel, pyruvate bolsters cytosolic energy state, thereby providing energy to maintain cellular functions in the face of metabolic challenges [10,29]. In addition, antioxidant actions of pyruvate could be pivotal to its cytoprotection. Pyruvate can directly degrade H_2O_2 and peroxynitrite in nonenzymatic reactions [13,30]. Consequently, H_2O_2 has a short half-life of only a few min when exposed to excess pyruvate. [14,31]. In this study, pyruvate exerted significant cytoprotection even when its administration was delayed until after the oxidative insult. Pyruvate provided essentially complete cytoprotection when administered concomitantly or 30 min after H_2O_2 , and still afforded significant, albeit incomplete, protection when given 1 or 2 hr after the oxidant. This delayed pyruvate cytoprotection suggested that mechanisms in addition to direct H_2O_2 degradation may have contributed to pyruvate's salutary effects. Indeed, a recently study also suggested that cytoprotection could be afforded even when pyruvate was provided 1–3 hr after H_2O_2 insult in primary cortical neurons [38]. Moreover, pyruvate protection is not limited to H_2O_2 -induced cytotoxicity, and has been demonstrated in response to various cytochemical insults, including zinc [27], menadion [13], NMDA [32], glutamate [42], β -amyloid [1], and 1-methyl-4-phenylpyridinium ion (MPP+) [33].

In this study, pyruvate's cytoprotective effects were examined by calcein AM and MTT assays. Reduction of the tetrazolium salt MTT to a blue formazan product is widely used for assessing cell survival. The reduction is mainly catalyzed by dehydrogenases localized in the mitochondria of viable cells [54]. Thus, the cytoprotection revealed by MTT assay suggested a direct action of pyruvate on mitochondria. Mitochondria are complex organelles involved in oxygen consumption, production of ATP, ROS generation and mobilization of calcium [19, 22,35]. Pyruvate has been reported to increase contribution of mitochondria to neuronal calcium homeostasis, and thereby mitigate glutamate-mediated neurotoxicity [42,45]. Accordingly, we tested the effects of pyruvate on cellular and mitochondrial ROS generation induced by oxidative stress.

Formation of ROS is an early cellular response to oxidative stress. Exposure to H_2O_2 initiated a rapid burst in ROS formation as demonstrated by the DCFH-DA assay. The fluorogenic compound DCFH-DA has been utilized extensively as a marker for overall intracellular

oxidative stress, and is thought to reflect the overall oxidative status of the cell [47]. DCFH is sensitive towards oxidation by H_2O_2 in combination with cellular peroxidases, peroxidases alone, peroxynitrite or $\cdot\text{OH}$, but is not suitable for measurement of nitric oxide, hypochlorous acid, or $\cdot\text{O}_2^-$ in biological systems [37]. In this study, pyruvate at low millimolar concentrations suppressed H_2O_2 -induced ROS formation. However, 100 μM pyruvate, although partially cytoprotective, did not affect intracellular ROS production in response to H_2O_2 . This discrepancy suggested other mechanisms may have mediated the cytoprotection by sub-millimolar concentrations of pyruvate.

Mitochondria are the major intracellular sources of ROS and also the major targets of oxidative stress [17]. We used DHR to examine the effect of pyruvate on mitochondrial ROS generation. DHR is the uncharged and nonfluorescent reduction product of the mitochondrial-selective dye rhodamine 123 and has been used to detect mitochondrial reactive oxygen and nitrogen species, including $\cdot\text{O}_2^-$ and peroxynitrite. Similar to its effects on the DCFH-DA assay, pyruvate also inhibited mitochondrial ROS generation induced by H_2O_2 . We further tested the effect of pyruvate on $\cdot\text{O}_2^-$ generation by submitochondrial particles. Inhibition of respiratory complexes I or III inhibition causes electrons to accumulate within respiratory chain components; these electrons can be added directly to oxygen molecules to produce $\cdot\text{O}_2^-$ [43, 44]. Here, inhibition of complexes I and III induced robust $\cdot\text{O}_2^-$ generation. Pyruvate not only inhibited $\cdot\text{O}_2^-$ production under basal conditions, but also suppressed $\cdot\text{O}_2^-$ generation induced by complex I or III inhibition.

The proposed mitochondria-protective effect of pyruvate was corroborated by its preservation of $\Delta\Psi_m$, the driving force for mitochondrial ATP production. Depolarization and collapse of $\Delta\Psi_m$ is one of the early events in the apoptotic cascade. Depolarization of $\Delta\Psi_m$ can be initiated by opening of mitochondrial permeability transition pores, followed by release of pro-apoptotic factors such as cytochrome c from the mitochondrial inter-membrane space. Pyruvate, a readily oxidized metabolic fuel, could bolster cytosolic energy state [29,31], thereby providing energy to maintain cellular functions in the face of oxidative stress [39]. Indeed, we found that pyruvate could maintain ATP production compromised by oxidative stress (data not shown). Pyruvate maintained $\Delta\Psi_m$, demonstrated by FRET assay and confocal microscopy of JC1, providing direct evidence to support the proposal that pyruvate protects neurons by stabilizing mitochondrial function.

In summary, the present study demonstrated that pyruvate exerts significant protection against oxidative stress in neuroblastoma SK-N-SH cells. Pyruvate suppresses mitochondrial ROS generation and maintains $\Delta\Psi_m$ under oxidative stress, indicating that mitochondria are the principal targets of pyruvate neuroprotection.

4. EXPERIMENTAL PROCEDURE

4.1. Chemicals

Sodium pyruvate, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), gentamycin, and H_2O_2 were purchased from Sigma (St. Louis, MO, USA). Calcein AM, 2,7-dichlorofluorescein diacetate (DCFH-DA), dihydrorhodamine 123 (DHR), nonyl acridine orange (NAO), and tetramethylrhodamine (TMR) were obtained from Molecular Probes (Eugene, OR, USA.). Fetal bovine serum (FBS) was purchased from Hyclone (Logan, UT, USA).

4.2. Cell Culturing

SK-N-SH human neuroblastoma cells were obtained from American Type Tissue Collection (Rockville, MD) at passage 38. Cells were grown to monolayer confluency in RPMI-1640

media supplemented with 10% FBS and 20 µg/ml gentamycin in plastic Nunc 75 cm² flasks (Fisher Scientific, Orlando, FL) at 37°C under 5% CO₂/95% air. Medium was changed 3 times/wk. Cells were observed with a phase-contrast microscope (Nikon Diaphot-300). SK-N-SH cells were back-cultured every 5–7 d using standard trypsinization procedures to maintain the cell line. SK-N-SH cells were used in passages 39–48. For oxidative insult, H₂O₂ was diluted with culture media to the desired concentration before use. Equimolar NaCl served as ‘vehicle’ control for sodium pyruvate.

4.3. Cell viability: calcein AM and MTT assays

For viability assay, SK-N-SH cells were plated at a density of 20,000 cells/well in 96-well plates 72 hr before initiation of experiments. Cells were exposed to treatments (H₂O₂, pyruvate) for 18 hr. For calcein AM assay, cells were rinsed with 1 X phosphate-buffered saline (PBS; pH 7.4) immediately after treatment, and calcein AM (25 µM) was added. After incubation for 15 min at 37°C, calcein AM fluorescence was determined at 485 nm excitation wavelength and 538 nm emission wavelength, using a Biotek FL600 microplate-reader (Highland Park, VT). Percentage viability was calculated by normalizing values to those of the H₂O₂-free control group, taken as 100% viable. For MTT assay, MTT (5 mg/ml) was added to cultures, incubated at 37°C for 3–4 hr, and then culture media was removed. Following overnight solubilization of the formazan product in 50% N,N-dimethyl formamide, 20% sodium dodecyl sulfate, pH 4.8, absorbance values were determined at 590 nm.

4.4. Measurement of ROS formation

The extent of cellular oxidative stress was assessed by monitoring the formation of free radical species using DCFH-DA or DHR. Cells were plated 72 hr before initiation of the experiment at a density of 20,000 cells/well in 96-well plates. For DCFH-DA assays, cells were incubated with 50 µM DCFH-DA for 45 min. After removal of DCFH-DA medium, cells were washed twice with 1 X PBS (pH 7.4) and incubated with RPMI containing 10% FBS and 150 µM H₂O₂ for 60 min. 2,7-dichlorofluorescein fluorescence was measured at 485 nm excitation wavelength and 538 nm emission wavelength. For DHR assay, cells were incubated with 10 µM DHR at 37°C for 30 min, then washed with PBS and incubated with 150 µM H₂O₂ as described above. DHR fluorescence was measured at 485 nm excitation wavelength and 538 nm emission wavelength. Fluorescence values were expressed as percentages of untreated control values.

4.5. Preparation of mitochondria and submitochondrial particles

SK-N-SH cells were harvested, resuspended in ice-cold mitochondria isolation buffer (0.3 M sucrose, 0.03 M nicotinamide, 0.02 M EDTA, pH 7.4) and homogenized with a teflon piston. Cell homogenates were centrifuged twice at 700 g for 10 min. The supernatant was collected and centrifuged at 10,000 g for 10 min. The pellet, *i.e.* the mitochondrial fraction, was resuspended in 30 mM potassium phosphate buffer (pH 7.4) and sonicated 4 times for 1 min with at least 1 min intervals between each sonication. Unbroken mitochondria were sedimented by centrifuging in mitochondrial isolation solution at 8000 g for 10 min. The supernatant containing the submitochondrial fraction was collected and centrifuged at 18,000 g for 60 min. The pellet, enriched in submitochondrial particles (SMP) was resuspended in 100 mM potassium phosphate buffer (pH 7.4). Protein concentrations were colorimetrically assayed as described by Bradford *et al.* [9], using bovine serum albumin at concentrations ranging from 0.063 to 1 mg/ml as standard.

4.6. Superoxide generation by submitochondrial particles

The rate of superoxide ($\cdot\text{O}_2^-$) generation was measured as previously described [52]. SMP were resuspended in 100 mM phosphate buffer (pH 7.4). The rate of SMP production of

$\cdot\text{O}_2^-$ was taken as the rate of SOD-inhibitable reduction of acetylated ferricytochrome c [3]. The reaction mixture contained 10 μM acetylated cytochrome c, 100 U SOD/ml, mitochondrial respiratory complex inhibitors (12 μM rotenone or 1.2 μM antimycin A) and 100 μg SMP protein in 100 mM potassium phosphate buffer (pH 7.4). The reaction was initiated by addition of 1 mM NADH. The rate of $\cdot\text{O}_2^-$ formation was measured by monitoring acetylated cytochrome c reduction at 550 nm ($\epsilon = 27.7 \text{ mM}^{-1}\text{cm}^{-1}$).

4.7. Mitochondrial membrane potential

Mitochondrial membrane potential (ψ_m) was monitored in intact cells by two methods: fluorescence resonance energy transfer assay (FRET) and confocal microscopy using JC1, a mitochondrial membrane potential dependent dye. The FRET assay is based on fluorescence resonance energy transfer between two dyes: nonyl acridine orange (NAO), which stains cardiolipin, a lipid found exclusively in the mitochondrial inner membrane, and tetramethylrhodamine (TMR), a potentiometric dye taken up by mitochondria in accord with Nernstian principles of potential and concentration. The presence of TMR quenches NAO emission in proportion to $\Delta\psi_m$, while loss of $\Delta\psi_m$, with consequent efflux of TMR, dequenches NAO. The high specificity of NAO staining, selective monitoring of the fluorescence emitted by NAO, not by TMR, and the stringent requirement for co-localization of both dyes within the mitochondrion, collectively enable the FRET assay to report $\Delta\psi_m$ without the confounding influence of background signal arising from potentiometric dye responding to plasma membrane potential. For FRET assay, cells were trypsinized and plated in clear-bottom, black-walled, 96-well plates at 60,000 cells / well (Costar 3606, Corning International, Corning, NY). Cells were loaded with 86 nM NAO for 5 min and washed 3 times with Hanks-buffered salt solution (HBSS), and then 80 μl of HBSS was added into each well followed by 20 μl TMR to a final concentration of 150 nM. After 5 min incubation, cells were exposed to 3 mM $\text{H}_2\text{O}_2 \pm 1 \mu\text{M}$ -1 mM sodium pyruvate. In control experiments, cells were exposed to neither H_2O_2 nor pyruvate. Mitochondrial membrane potential collapse was measured by monitoring NAO fluorescence at 488 nm excitation / 525 nm emission wavelengths.

For confocal microscopy, cells were seeded on 25 mm cover slips at a density of 600,000 cells / well, grown for 24 hr, and then incubated with JC1 (10 $\mu\text{g}/\text{ml}$) for 2 hr. The cover slip was washed twice and mounted in a cell chamber (ALA Scientific Instruments, Westbury, NY). Serial confocal images were taken every 10 min with a confocal microscope with excitation at 490 nm and emission at 510 and 590 nm. Mitochondrial membrane depolarization was induced by adding H_2O_2 to a final concentration of 3 mM, with co-treatment with pyruvate (2 mM) or control.

4.8. Data Analysis

Data in the figures are presented as mean values $\pm$ SEM. Sodium pyruvate-treated and control groups were compared using one-way ANOVA with Tukey's multiple-comparisons test. For all tests, P values < 0.05 were considered statistically significant.

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Abbreviations

ROS	reactive oxygen species
SMPs	submitochondrial particles
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
DCFH-DA	2,7-dichlorofluorescein diacetate
DHR	dihydrorhodamine 123
NAO	nonyl acridine orange
TMR	

	tetramethylrhodamine
FRET	fluorescence resonance energy transfer
$\Delta\Psi_m$	mitochondrial membrane potential
PBS	phosphate-buffered saline
HBSS	Hanks-buffered salt solution
H_2O_2	hydrogen peroxide
FBS	fetal bovine serum

Figure 1. Cytoprotective effect of pyruvate against H₂O₂-induced cell death

Cell viability was determined by calcein AM assay. H₂O₂ (150 μM) exposure for 18 hr induced 85% cell death. Co-treatment of sodium pyruvate dose-dependently increased cell survival (panel A). Delayed pyruvate administration 30 min (panel B), 1 hr (panel C) and 2 hr (panel D) after H₂O₂ also afforded significant protection against oxidative insult. Values are means

± SEM from 8 experiments per group. In this figure and Figures 2–5, each experiment was replicated at least 3 times. *** $p < 0.001$ vs H_2O_2 + vehicle.

Figure 1A. Co-treatment of pyruvate provides cytoprotection against hydrogen peroxide insult.

Figure 1B. Thirty minute post-treatment of pyruvate provides cytoprotection against hydrogen peroxide insult.

Figure 1C. Sixty minute post-treatment of pyruvate provides cytoprotection against hydrogen peroxide insult.

Figure 1D. Two hour post-treatment of pyruvate provides cytoprotection against hydrogen peroxide insult.

Figure 2. Cytoprotective effect of pyruvate against H₂O₂ insult

Cell viability was assessed by MTT assay. Exposure to 150 μ M H₂O₂ for 18 hr markedly decreased MTT reduction to 37.0 $\pm$ 0.8 % of baseline. Pyruvate co-treatment at $\geq$ 0.1 mM ameliorated H₂O₂-induced loss of MTT reduction. Values are means $\pm$ SEM from 8 experiments per group. ** p < 0.01 vs H₂O₂ + vehicle; ***p < 0.001 vs H₂O₂ + vehicle. Figure 2. Cytoprotective effect of pyruvate against hydrogen peroxide insult as determined by MTT assay.

Figure 3. Pyruvate suppression of hydrogen peroxide-induced intracellular (panel A) and mitochondrial (panel B) ROS formation

Panel A: 150 μM H₂O₂ increased intracellular ROS accumulation, indicated by DCF fluorescence, which plateaued at 45–60 min exposure. Pyruvate co-treatment at 1 and 2 mM, but not at sub-millimolar concentrations, sharply lowered intracellular ROS formation. Panel B: 150 μM H₂O₂-induced mitochondrial ROS production, assessed from DHR fluorescence, plateaued at 30 min exposure. Pyruvate (1–4 mM) concentration-dependently suppressed mitochondrial ROS formation induced by H₂O₂. Values are means ± SEM from 8 experiments per group. **p* < 0.05 vs H₂O₂ + vehicle, *** *p* < 0.001 vs H₂O₂ + vehicle.

Figure 3A. Pyruvate decrease hydrogen peroxide-induced intracellular free radical production as determined by DCFH-DA.

Figure 3B. Pyruvate decrease hydrogen peroxide-induced mitochondrial ROS production as determined by DHR.

Figure 4. Pyruvate attenuates $\cdot\text{O}_2^-$ formation by submitochondrial particles in absence (panel A) and presence (panels B, C) of respiratory complex inhibitors

Rotenone, a respiratory complex I inhibitor (panel B) and antimycin D, a complex III inhibitor (panel C) increased $\cdot\text{O}_2^-$ formation over the basal, inhibitor-free rate (panel A). Basal and respiratory inhibitor-enhanced $\cdot\text{O}_2^-$ formation was suppressed by 0.1 and 1 mM pyruvate. Values are means $\pm$ SEM from 4–5 experiments per group. * $p < 0.05$ vs control, *** $p < 0.001$ vs control.

Figure 4A. Pyruvate decreases superoxide production by SMP.

Figure 4B. Pyruvate decreases superoxide production by SMP induced by complex I inhibitor rotenone.

Figure 4C. Pyruvate decrease superoxide production by SMP induced by complex III inhibitor antimycin A.

Figure 5. Pyruvate mitigates H₂O₂-induced mitochondrial depolarization

Panel A: Mitochondrial membrane potential collapse induced by 3 mM H₂O₂ and the effect of pyruvate. H₂O₂ caused $\Delta\Psi_m$ collapse, evidenced by the increase of NAO fluorescence as the consequence of efflux of TMR. Pyruvate dose-dependently attenuated H₂O₂-induced mitochondrial depolarization. Values are means $\pm$ SEM from 6–8 experiments. ** $p < 0.01$ vs vehicle, *** $p < 0.001$ vs vehicle. Panel B: Confocal microscopic images of mitochondrial membrane depolarization induced by hydrogen peroxide and the effect of pyruvate. Confocal microscopic images show the same field of cells viewed before and at 10, 20, and 30 min after H₂O₂ (2 mM) exposure with vehicle (upper) or 2 mM sodium pyruvate (lower) co-treatment. After exposure to H₂O₂, aggregated JC1 (in red) became monomeric and fluoresced green upon mitochondrial depolarization, as evidenced by the increase of yellow (merging of red and green) in the time series images. Pyruvate co-treatment minimized mitochondrial depolarization induced by H₂O₂.

Figure 5A. Mitochondrial membrane potential collapse induced by hydrogen peroxide and the effect of pyruvate as determined by FRET assay.

Figure 5B. Confocal microscopic images of mitochondrial membrane potential collapse induced by hydrogen peroxide and the effect of pyruvate.