Pyruvate Protects Neurons against Hydrogen Peroxide-Induced Toxicity

Solange Desagher, Jacques Glowinski, and Joël Prémont

Chaire de Neuropharmacologie, Institut National de la Santé et de la Recherche Médicale U114, Collège de France, 75 231 Paris Cedex 05, France

Hydrogen peroxide (H_2O_2) is suspected to be involved in numerous brain pathologies such as neurodegenerative diseases or in acute injury such as ischemia or trauma. In this study, we examined the ability of pyruvate to improve the survival of cultured striatal neurons exposed for 30 min to H_2O_2, as estimated 24 hr later by the 3-[4,5-dimethylthiazol-2-yl]−2,5-diphenyltetrazoliumbromide assay. Pyruvate strongly protected neurons against both H_2O_2 added to the external medium and H_2O_2 endogenously produced through the redox cycling of the experimental quinone menadione. The neuroprotective effect of pyruvate appeared to result rather from the ability of α-ketoacids to undergo nonenzymatic decarboxylation in the presence of H_2O_2 than from an improvement of energy metabolism. Indeed, several other α-ketoacids, including α-ketobutyrate, which is not an energy substrate, reproduced the neuroprotective effect of pyruvate. In contrast, lactate, a neuronal energy substrate, did not protect neurons from H_2O_2. Optimal neuroprotection was achieved with relatively low concentrations of pyruvate (≤1 mM), whereas at high concentration (10 mM) pyruvate was ineffective. This paradox could result from the cytosolic acidification induced by the cotransport of pyruvate and protons into neurons. Indeed, cytosolic acidification both enhanced the H_2O_2-induced neurotoxicity and decreased the rate of pyruvate decarboxylation by H_2O_2. Together, these results indicate that pyruvate efficiently protects neurons against both exogenous and endogenous H_2O_2. Its low toxicity and its capacity to cross the blood–brain barrier open a new therapeutic perspective in brain pathologies in which H_2O_2 is involved.

Key words: pyruvate; α-ketoacids; antioxidants; hydrogen peroxide; menadione; oxidative stress; neuroprotection; neurotoxicity

Oxygen-derived free radical generation has been implicated in the etiology of some neurodegenerative diseases (Olanow, 1993; Simonian and Coyle, 1996) and in neuronal death after acute injury such as ischemia-reperfusion or trauma (Siesjö et al., 1989; Traystman et al., 1991). In particular, superoxide anion ($O_2^{\cdot -}$), which has limited toxic effects in itself, can either react with nitric oxide to form peroxinitrite anions, which are highly cytotoxic, or dismutate into hydrogen peroxide (H_2O_2), a reaction that is accelerated by superoxide dismutase. In turn, H_2O_2 can exert its toxic effects mainly through the ferrous iron-dependent formation of the highly reactive hydroxyl radical ($OH^{\cdot}$) (Fenton, 1894), which leads to alterations of lipids, proteins, and DNA (Halliwell, 1992). Probably of less importance, the modification of the redox thiol status of the cytosol could also contribute to H_2O_2 toxicity.

Under pathological situations such as ischemia-reperfusion, various cell types including neurons produce large amounts of H_2O_2. Because of its high membrane permeability (Halliwell, 1992), H_2O_2 can be cytotoxic not only for the producing cell but also for neighboring cells. As generally accepted, the enzymatic cellular defense against H_2O_2 includes catalase and glutathione peroxidase (Simonian and Coyle, 1996). We have recently reported that catalase plays a predominant protecting role against H_2O_2 within astrocytes, whereas glutathione peroxidase is the main protective enzyme in neurons (Desagher et al., 1996).

However, nonenzymatic mechanisms can also contribute to the cellular defense against H_2O_2-induced cytotoxicity. Indeed, pyruvate and other α-ketoacids, abundantly present in mammalian cells, can react nonenzymatically with H_2O_2. Through this reaction first described by Holleman (1904), carbon dioxide is liberated, and the α-ketoacid is converted into the corresponding carboxylic acid: $R - CO\,COOH + H_2O_2 \rightarrow R - COOH + CO_2 + H_2O$.

Pyruvate can be transported into or secreted from the cells by the specific H^+-monocarboxylate cotransporter (Poole and Halestrap, 1993; Garcia et al., 1994). It could thus act as both intracellular and extracellular H_2O_2 scavengers. The present study was undertaken to determine whether pyruvate and other α-ketoacids can indeed protect neurons against H_2O_2. Cultured striatal neurons from mouse embryos were exposed to either exogenous H_2O_2 or to H_2O_2 intracellularly formed as a result of the use of menadione (2-methyl-1,4-naphthoquinone), a quinone that generates intracellular $O_2^{\cdot -}$ and H_2O_2, through a redox cycling process (Thor et al., 1982; Doroshow, 1986). We demonstrate that pyruvate protects neurons against both exogenous and endogenously produced H_2O_2.

MATERIALS AND METHODS

Materials. Swiss mice were obtained from Iffa Credo (Lyon, France); PBS without calcium and magnesium and culture media were from Life Technologies (Gaithersburg, MD); fetal calf serum was from Dutcher (Brumath, France); 5- and 6-carboxy-SNARF-1 AM acetate (lot 2651-4) was from Molecular Probes Europe BV (Leiden, The Netherlands); water, [^{3}H]carboxyl (1.0 mCi/g), and inulin-carboxyl [carboxyl-^{14}C] (2.2

Received June 6, 1997; revised Sept. 17, 1997; accepted Sept. 23, 1997.

This study was supported by Institut National de la Santé et de la Recherche Médicale, Direction des Recherches Etudes et Techniques Grant 94158, and Rhône-Poulenc-Rorer.

Correspondence should be addressed to Solange Desagher, Chaire de Neuropharmacologie, Institut National de la Santé et de la Recherche Médicale U114, Collège de France, 11 place Marcelin Berthelot, 75 231 Paris Cedex 05, France.

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mCi/g) were from DuPont NEN; horseradish peroxidase, catalase (bovine liver), L-lactic acid dehydrogenase (LDH, type XI from rabbit muscle), *o*-dianisidine (3,3′-dimethoxybenzidine), β-nicotinamide adenine dinucleotide reduced form (β-NADH) disodium salt, H_2O_2, pyruvic acid sodium salt, L-(+)-lactic acid sodium salt, α-ketoglutaric acid monosodium salt, β-ketoglutaric acid, oxaloacetic acid, α-ketobutyric acid sodium salt, menadione (2-methyl-1,4-naphthoquinone) sodium bisulfite, *N*-morpholinopropanesulfonic acid, 2-deoxy-D-glucose, 3-[4,5-dimethylthiazol-2-yl]−2,5-diphenyltetrazoliumbromide (MTT), phloretin, and all other chemicals or reagents used in the present study were purchased from Sigma (Saint Quentin Fallavier, France).

Primary culture of striatal neurons. Primary neuronal cultures were prepared using the method of El Etr et al. (1989) with slight modifications. Briefly, striata were removed from 14- to 15-d-old Swiss mouse embryos and mechanically dissociated with a flame-narrowed Pasteur pipette in PBS supplemented with glucose (33 mM). Cells were plated on 24-well Nunc (Roskilde, Denmark) culture dishes (5×10^5 cells per well containing 0.5 ml of medium) or 50 mm Nunc petri dishes (5×10^6 cells per dish in 5 ml), previously and successively coated with poly-L-ornithine (15 μg/ml; M_r 40 kDa), and the culture medium containing 10% fetal calf serum. After the removal of the last coating solution, cells were seeded in a serum-free medium consisting of a 1:1 mixture of DMEM and Ham's F12 nutrient, supplemented with glucose (33 mM), glutamine (2 mM), $NaHCO_3$ (13 mM), HEPES buffer (5 mM, pH 7.4), penicillin–streptomycin (5 IU/ml and 5 μg/ml, respectively), and a mixture of salt and hormones containing insulin (25 μg/ml), transferrin (100 μg/ml), progesterone (20 nM), putrescine (60 μM), and sodium selenite (30 nM). Cells were cultured at 37°C in a humidified atmosphere of 92% air and 8% CO_2. After 1 week in culture, cells were immunocytochemically defined according to the method of El Etr et al. (1989) as purified neurons devoid of detectable glial elements. Neurons were used at 6–7 d *in vitro*.

Neurotoxicity experiments. Neurons were first washed with Krebs' bicarbonate buffer (in mM: 124 NaCl, 3.5 KCl, 1.25 K_2HPO_4, 26.3 $NaHCO_3$, 1.2 $CaCl_2$, 1.2 $MgSO_4$, and 11 glucose, equilibrated with 92% air and 8% CO_2 at 37°C, pH 7.4) or preincubated for indicated times in the presence of different agents. Cells were then incubated in the same buffer at 37°C in a humidified atmosphere (92% air and 8% CO_2) with H_2O_2 or menadione for the indicated time. After the incubation period, cells were washed with Krebs' bicarbonate buffer and cultured for another period of 24 hr in the initial culture medium previously stored.

To investigate the influence of intracellular acidification on the neurotoxic effect of H_2O_2, neurons were successively preincubated and incubated with H_2O_2 in a buffer containing (in mM): 130 NaCl, 5 KCl, 1 $CaCl_2$, 1.2 $MgCl_2$, 20 HEPES, and 11 glucose, adjusted to the indicated pH with NaOH.

MTT colorimetric assay. Previously, we estimated the survival of striatal neurons 24 hr after the exposure to H_2O_2 by two different methods: the MTT assay and an ELISA with antibodies directed against an antigen specifically located in neurons (microtubule-associated protein-2) (Desagher et al., 1996). The two methods gave exactly the same results. Therefore, the more convenient and rapid method, i.e., the MTT colorimetric assay, was used in the present study.

This method is based on reduction of the tetrazolium salt MTT into a crystalline blue formazan product by the cellular oxidoreductases (Slater et al., 1963; Berridge and Tan, 1993). Therefore, the amount of formazan produced is proportional to the number of viable cells. Briefly, the culture medium was replaced by a solution of MTT (0.5 mg/ml) in PBS supplemented with glucose (33 mM). After a 3 hr incubation at 37°C, this solution was removed, and the produced blue formazan was solubilized in 1 ml of pure dimethyl sulfoxide. The optical density of the formed blue formazan was measured at 560 nm.

Determination of H_2O_2 concentration. The concentrations of H_2O_2 were estimated with a colorimetric assay using *o*-dianisidine (3,3′-dimethoxybenzidine). This compound, which is colorless in its reduced form, is oxidized in the presence of H_2O_2 and peroxidase into a red product. The sample was added to 0.5 mM *o*-dianisidine and 60 IU/ml horseradish peroxidase. H_2O_2 reacted instantaneously and totally with *o*-dianisidine. Optical density was estimated at 500 nm. The concentrations of H_2O_2 were determined using standard solutions.

Fluorimetric determination of intracellular pyruvate content. After different treatments, neurons, cultured in 50 mm dishes, were rapidly washed twice with ice-cold PBS containing 100 μM phloretin, an inhibitor of the monocarboxylate transporter (Poole and Halestrap, 1993), and all the buffer was carefully aspirated. Cells were incubated in 500 μl of 0.25 M ice-cold perchloric acid for 5 min, scraped with a rubber policeman, and centrifuged at 12,000 × *g* for 5 min to remove proteins. The supernatant was neutralized with a solution containing 2 M KOH and 0.3 M *N*-morpholinopropanesulfonic acid. Catalase (500 IU/ml) was then added to ensure the stability of pyruvate. After centrifugation and elimination of the potassium perchlorate formed, the samples were stored at −20°C before use.

The fluorimetric assay was a modification of the method of Lowry and Passonneau (1972). Pyruvate and NADH were respectively converted into lactate and NAD by LDH. The decrease of NADH fluorescence (excitation, 340 nm; emission, 460 nm) was followed by a Hitachi F-2000 fluorescence spectrophotometer. Each sample was mixed with Tris-HCl buffer (100 mM, pH 7.4) containing an NADH concentration that exceeded the expected pyruvate concentration at least fivefold. The reaction was started by adding LDH (4.6 IU/ml), and the fluorescence was followed until no further change was observed. The concentrations of pyruvate were determined using standard solutions.

In each assay, the protein content was determined in 50 mm dishes of sister cultures by the method of Bradford (1976) with bovine serum albumin as the standard. The intracellular water volume of cultured neurons was estimated according to the method of Rottenberg (1979) by the use of tritiated water and [^{14}C]inulin.

Cytosolic pH measurements. For measurements of intracellular pH (pHi), cells were cultured on glass slides (6×10^6 cells per slide), coated successively with poly-L-ornithine (15 μg/ml) and culture medium supplemented with 10% fetal calf serum and 1 μg/ml laminin, and placed into 100 mm culture dishes. Intracellular pH was monitored by quantitative ratio imaging of the fluorescent dye 5- and 6-carboxy SNARF-1 AM acetate (Whitaker et al., 1991). Cells were loaded for 45 min with 17 μM SNARF-1 AM in perfusion buffer (in mM: 20 HEPES, 5.5 glucose, 120 NaCl, 5 KCl, 1 $MgCl_2$, and 1.2 $CaCl_2$, pH 7.4). After loading, the glass slide was placed in a perfusion chamber where cells were exposed to tested substances using a multichannel superfusion device. Cells were excited with a 75 W Xenon light, filtered at 535 nm with a 10-nm-wide interferential filter. Excitation and emission spectra were separated by a 560 nm dichroic long-pass filter, and the emission spectra were divided in two halves (opticals were obtained from Nikon and Hamamatsu). Two discriminant bands were selected from the two halves at 580 and 640 nm, and both fluorescent images were digitized (eight video frames per digitized image, permitting the recording of one image per second). The camera dark noise was substracted from the recorded crude image (camera and digitizing system were from Hamamatsu). Fields for imaging were selected under bright-field illumination before any fluorescence measurement and contained 7–21 healthy, intact neurons. *In situ* calibration of pHi, measured with SNARF-1, was performed by exposing cells to nigericin (10 μM), along with high K^+ (100 mM) buffers (Thomas et al., 1979) ranging from pH 6.5 to 7.5.

RESULTS

Pyruvate protects neurons from the toxicity induced by exogenous H_2O_2

Cultured striatal neurons were incubated for 30 min with increasing concentrations of H_2O_2 in the absence or presence of 2 mM sodium pyruvate. Pyruvate (2 mM) completely protected neurons from the H_2O_2-induced toxicity up to 300 μM (Fig. 1), an H_2O_2 concentration higher than that measured during the ischemia-reperfusion period (Hyslop et al., 1995). The neuroprotective effect of pyruvate was only partial when H_2O_2 was added at 1 mM (Fig. 1). When exposed to 200 μM H_2O_2, striatal neurons were progressively protected by increasing concentrations of pyruvate. This neuroprotection was already significant for a pyruvate concentration of 0.4 mM and almost complete at 2 mM (Fig. 1). The same neuroprotective effects of pyruvate were observed if neuronal survival was measured using ELISA with antibodies directed against microtubule-associated protein-2 (not shown).

Pyruvate protects neurons from the toxicity induced by menadione

The capacity of pyruvate to protect neurons against endogenously produced H_2O_2 was investigated with the use of menadione. Like other quinones, menadione can enter flavoprotein-catalyzed re-

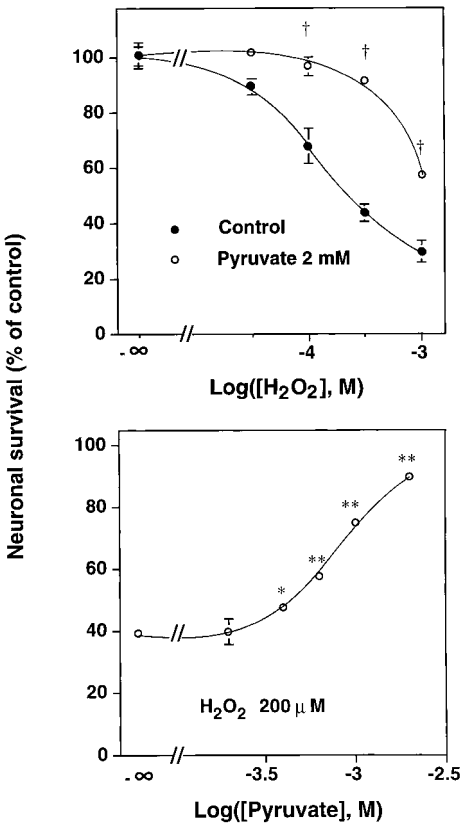

Figure 1. Pyruvate protects neurons from exogenous H_2O_2-induced toxicity. Primary cultures of striatal neurons were preincubated in Krebs' bicarbonate buffer at 37°C for 30 min with 2 mM (*top*) or increasing concentrations (*bottom*) of sodium pyruvate and then further incubated for 30 min with increasing concentrations of H_2O_2 (*top*) or with 200 μM H_2O_2 (*bottom*) in the presence or absence of the indicated concentration of pyruvate. Pyruvate and H_2O_2 were simultaneously applied to the cells. Neuronal survival was estimated 24 hr later by the MTT colorimetric assay. Results are expressed as the percentage of surviving neurons compared with control cultures. Data are the mean ± SEM of three independent experiments, each performed on triplicate wells. When not visible, the sizes of the error bars are less than those of the symbols. $^{\dagger}p < 0.001$; significantly different from the corresponding values determined in the absence of pyruvate (ANOVA followed by Bonferroni's test). $^{*}p < 0.05$; $^{**}p < 0.01$; significantly different from the value obtained in the absence of pyruvate (ANOVA followed by Dunnett's test).

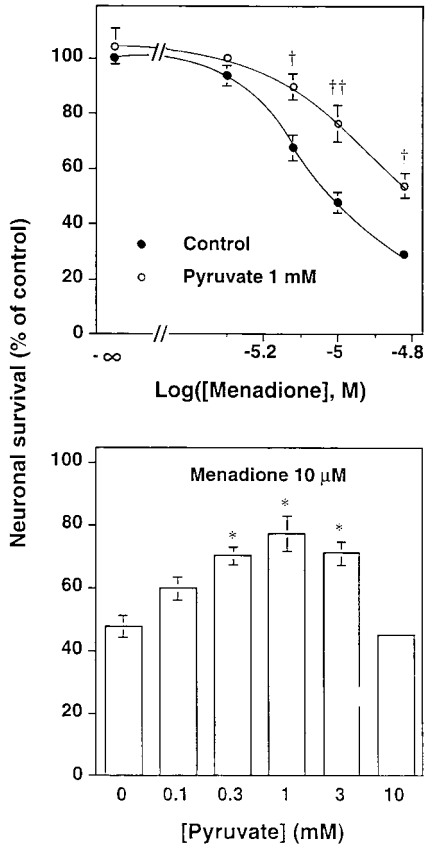

Figure 2. Pyruvate partly protects neurons from menadione-induced toxicity. Primary cultures of neurons were incubated in Krebs' bicarbonate buffer at 37°C for 1 hr with increasing concentrations of menadione in the absence or the presence of 1 mM sodium pyruvate (*top*) or with 10 μM menadione in the presence of increasing concentrations of pyruvate (*bottom*). Cells were then washed and further incubated for 30 min with or without pyruvate and replaced into the initial culture medium supplemented with the corresponding concentrations of pyruvate. Neuronal survival was estimated 24 hr later. Results are expressed as the percentage of surviving neurons compared with control cultures not treated with menadione. Data are the mean ± SEM of three independent experiments, each performed on triplicate wells. $^{\dagger}p < 0.01$; $^{\dagger\dagger}p < 0.001$; significantly different from the corresponding values determined in the absence of pyruvate (ANOVA followed by Bonferroni's test). $^{*}p < 0.01$; significantly different from the value obtained in the absence of pyruvate (ANOVA followed by Dunnett's test).

dox cycles with molecular oxygen, and this results in the formation of large amounts of $O_2^{\cdot -}$ (Thor et al., 1982). Because of the subsequent dismutation of $O_2^{\cdot -}$, toxic concentrations of H_2O_2 are then intracellularly formed, as demonstrated by the direct estimation of H_2O_2 released from neurons (Nath et al., 1995; Desagher et al., 1996).

Exposure of striatal neurons to increasing concentrations of menadione (5–15 μM) for 1 hr induced a progressive cell death that was significantly reduced by 1 mM sodium pyruvate (Fig. 2). This protective effect of pyruvate was observed if the α-ketoacid was added in the incubation medium and in the culture medium for 24 hr after menadione exposure. Indeed, the quinone produced H_2O_2 in the cells even after this compound was removed from the incubation medium. The pyruvate protection was significant for all neurotoxic concentrations of menadione (Fig. 2). Conversely, in the presence of 10 μM menadione, increasing concentrations of pyruvate (up to 1 mM) produced a progressive enhancement of the neuronal survival observed 24 hr later (Fig. 2). The lack of protection observed with the highest concentration of pyruvate tested (10 mM) might be attributable to the intracellular acidification that counteracts the beneficial effect of pyruvate (see below). The addition of 10 mM sodium pyruvate alone did not significantly change the cell viability (not shown).

Catalase reduced the neurotoxicity of menadione. This result suggests that H_2O_2 released in the extracellular medium contributes to the toxic effect of the quinone through a paracrine mechanism (33 ± 2 and 61 ± 2% of neuronal survival estimated 24 hr after exposure to 10 μM menadione for 1 hr in the absence or the presence of 500 IU/ml catalase, respectively, mean ± SEM, from three independent experiments performed in triplicates). However, at optimal concentration (1 mM), pyruvate was found to be more efficient than catalase to protect neurons (Fig. 2).

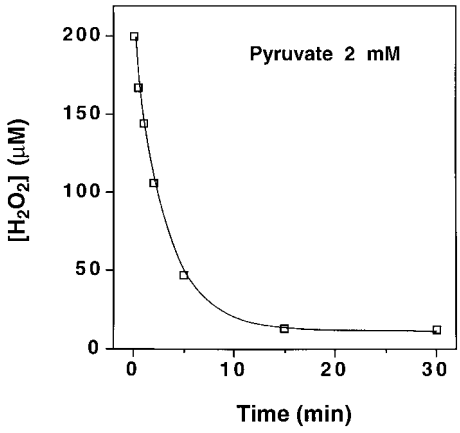

Figure 3. Kinetics of the reaction of H_2O_2 and pyruvate in the absence of cells. Pyruvate (2 mM) and H_2O_2 (200 μM) were mixed in Krebs' bicarbonate buffer at 37°C in the absence of cells. The residual concentrations of H_2O_2 were determined at indicated times as described in Materials and Methods. Data are the mean ± SEM of three independent experiments each performed in triplicate. The error bars are not visible, because they are smaller than the symbols.

Mechanisms involved in the neuroprotective effects of pyruvate and other α-ketoacids against H_2O_2 neurotoxicity

As already indicated (see the introductory remarks), the neuroprotective effect of pyruvate against H_2O_2 toxicity may be attributable to its ability to degrade H_2O_2 through a nonenzymatic oxidative decarboxylation, leading to the formation of carbon dioxide, water, and acetate (Holleman, 1904; Bunton, 1949). This reaction may occur both in the extracellular and intracellular medium, leading to the degradation of equal amounts of H_2O_2 and pyruvate. However, to be neuroprotective, pyruvate must react with H_2O_2 before the formation of $OH^{\bullet}$ and the subsequent appearance of irreversible damage.

Therefore, to determine whether the degradation rate of H_2O_2 by pyruvate is compatible with its neuroprotective effect, the rate of the reaction was estimated using respective concentrations of pyruvate and H_2O_2, leading to an almost complete neuronal protection (Fig. 3). Pyruvate (2 mM) and H_2O_2 (200 μM) were mixed in Krebs' bicarbonate buffer at 37°C, and H_2O_2 levels were estimated at various times after the onset of the reaction. In this condition, half of the H_2O_2 initially present remained in the medium after 2 min, and H_2O_2 levels became negligible after 15 min (Fig. 3). Therefore, cultured neurons were exposed to high levels of H_2O_2 only during the first 5 min, and as demonstrated previously, this is insufficient to induce significant cell death (Desagher et al., 1996). Pyruvate may thus protect neurons against H_2O_2-induced toxicity by reacting with the oxidant. The rate of the reaction might account also for the reduced protective effect of pyruvate when the $[H_2O_2]$/[pyruvate] ratio was increased (Fig. 1). Sodium acetate (200 μM), the decarboxylation product of sodium pyruvate, did not significantly modify neuronal viability either in control conditions or in the presence of 200 μM H_2O_2 (Table 1).

Complementary experiments were performed to determine whether pyruvate could also protect neurons against H_2O_2 toxicity by improving energy metabolism. For this purpose, striatal neurons were exposed for 30 min to H_2O_2 (200 μM) in the presence of α-ketoglutarate and oxaloacetate, which are known to act both as H_2O_2 scavengers and energy substrate metabolites,

Table 1. Effect of sodium acetate on neuronal survival

Treatment	Neuronal survival (% of control)
Control	100 ± 2.4
Acetate (200 μM)	105.9 ± 0.5NS
H_2O_2 200 (μM)	46.9 ± 2.8
H_2O_2 (200 μM) + acetate (200 μM)	54.8 ± 2.2NS

Neurons were preincubated for 30 min with or without 200 μM sodium acetate and further incubated for 30 min with 200 μM H_2O_2 in either the presence or absence of sodium acetate. Neuronal survival was estimated 24 hr later. Results are expressed as the percentage of surviving neurons compared with control cultures. Data are the mean ± SEM of three independent experiments performed in triplicate. NS, Not significantly different from the corresponding values obtained in the absence of acetate (ANOVA followed by Bonferroni's test).

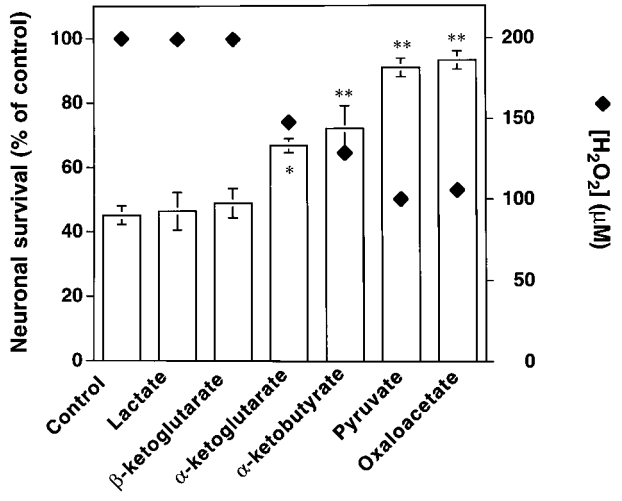

Figure 4. H_2O_2-scavenging capacities and neuroprotecting properties of various α-ketoacids. A 200 μM concentration of H_2O_2 was incubated with 2 mM sodium lactate, β-ketoglutarate, α-ketoglutarate, α-ketobutyrate, pyruvate, or oxaloacetate in Krebs' bicarbonate buffer for 2 min at 37°C in the absence of cells. The residual concentration of H_2O_2 (*filled symbols*) was determined in each experimental condition. The error bars are not visible, because they are smaller than the symbols. In a separate set of experiments, cultured neurons were preincubated for 30 min with a 2 mM concentration of each compound and further incubated for 30 min with 200 μM H_2O_2 in their presence or absence. Neuronal survival was estimated 24 hr later. Results are expressed as the percentage of living neurons compared with cultures not treated with H_2O_2. Data are the mean ± SEM of three independent experiments each performed in triplicate. $^*p < 0.05$; $^{**}p < 0.01$; significantly different from the control value (ANOVA followed by Dunnett's test).

α-ketobutyrate, which only possesses H_2O_2 scavenger properties, lactate, which is only an energy substrate metabolite (Schurr et al., 1988), or finally β-ketoglutarate, which is neither an H_2O_2 scavenger nor an energy substrate metabolite. These compounds were all added at a concentration of 2 mM.

As shown in Figure 4, the ability of these different compounds to prevent H_2O_2 toxicity was related to their capacity to scavenge H_2O_2 and completely independent of their ability to be used as energy substrates. In particular, lactate was ineffective, whereas oxaloacetate strongly prevented H_2O_2 toxicity. In addition, the ability of the different α-ketoacids (used at the same concentration, 2 mM) to scavenge H_2O_2 was closely correlated with their capacity to protect neurons: oxaloacetate = pyruvate > α-ketoglutarate = α-ketobutyrate (which is not an energy substrate metabolite). As lactate, β-ketoglutarate was ineffective (Fig. 4).

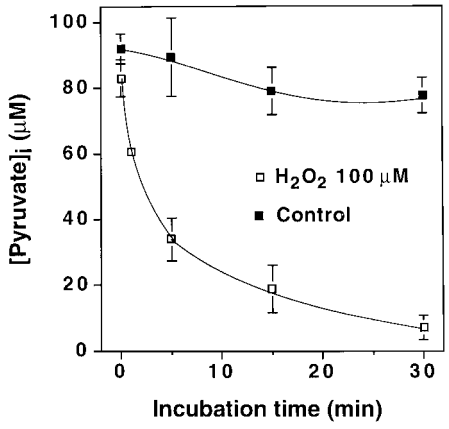

Figure 5. Exogenous H_2O_2 decreased intracellular pyruvate. Striatal neurons were incubated in Krebs' bicarbonate buffer with or without 100 μM H_2O_2 for indicated times. Then, neuronal cultures were washed with 500 IU/ml catalase for 30 sec. Cells were subsequently treated as indicated in Materials and Methods. The cytosolic neuronal volume and the residual intracellular pyruvate were measured as described in Materials and Methods. Data are the mean ± SEM of three independent experiments, each performed in triplicate.

H_2O_2 decreased the cellular pyruvate content

Conversely, because of its high membrane permeability (Halliwell, 1992), H_2O_2 may depress energy metabolism through the degradation of intracellular pyruvate (and other α-ketoacids). Supporting this hypothesis, intracellular levels of pyruvate were markedly reduced when striatal neurons were exposed to 100 μM H_2O_2 (Fig. 5). Indeed, pyruvate levels reached already one-third of basal levels within 5 min and became negligible after 30 min, whereas intracellular levels of pyruvate were only slightly reduced under control conditions (Fig. 5).

Influence of pH on the neuroprotective effect of pyruvate

Three observations suggest that high concentrations of pyruvate can counteract its neuroprotective effects by inducing an intracellular acidification.

First, pyruvate, as lactate, is transported across the plasma membrane by the H^+-monocarboxylate cotransporter (Poole and Halestrap, 1993). As shown in Figure 6, external pyruvate was rapidly transported into neurons, an equilibrium being reached between the external and internal concentrations in 5 min. Accordingly, exposure of neurons to 10 mM pyruvate resulted in a sustained cytosolic acidification as measured with the use of the proton-sensitive dye carboxy SNARF-1 (Fig. 7).

Second, the rate of H_2O_2 degradation by pyruvate was reduced when the pH was decreased from 7.4 to 5.4 (Fig. 8).

Third, intracellular acidification potentiated H_2O_2-induced neuronal death (Fig. 8). Indeed, the neurotoxicity induced by 30 μM H_2O_2 was strongly increased when extracellular pH (pHe) was decreased. It has been reported that pHi reaches approximately the pHe level by 10 min (Nedergaard et al., 1991). Intracellular acidification alone did not significantly alter the survival of striatal neurons except for pH 5.4 (Fig. 8).

Together, these results indicate that intracellular acidification induced by high concentrations of pyruvate not only moderates the scavenging capacity of pyruvate but also potentiates H_2O_2 neurotoxicity. The lack of protection of 10 mM pyruvate against menadione-induced toxicity might be the illustration of this paradox (Fig. 2).

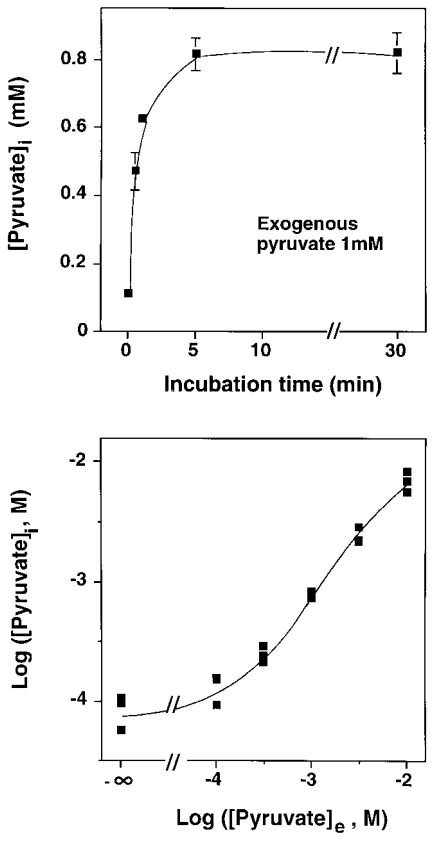

Figure 6. Pyruvate uptake by striatal neurons. Primary cultures of striatal neurons were incubated in Krebs' bicarbonate buffer at 37°C with 1 mM sodium pyruvate for the indicated times (*top*) or with increasing concentrations of pyruvate ($[Pyruvate]_e$) for 10 min (*bottom*). Neurons were treated, and intracellular concentrations of pyruvate ($[Pyruvate]_i$) were determined as described in Materials and Methods. *Top*, Data are the mean ± SEM of two independent experiments, each performed in triplicate. *Bottom*, Individual results of three independent experiments, also performed in triplicate.

DISCUSSION

The present study demonstrates that extracellular pyruvate protects neurons against the neurotoxicity induced by exogenous or endogenously produced H_2O_2.

The antioxidant protective effect of α-ketoacids has already been investigated both *in vitro* in several cell types (Andrae et al., 1985; O'Donnell-Tormey et al., 1987) and *in vivo* in whole organs such as heart or kidney (Cavallini et al., 1990; Salahudeen et al., 1991; Crestanello et al., 1995). However, to our knowledge, this process has never been investigated in neuronal cells. Our results indicate that pyruvate and related α-ketoacids improve the survival of cultured neurons exposed to H_2O_2. Several observations suggest that the protective effect of pyruvate results rather from its ability to react with H_2O_2 to form acetate, water, and carbon dioxide than from the improvement of neuronal energy metabolism: (1) the neuroprotective effect of pyruvate was reproduced by several α-ketoacids, which share with pyruvate the ability to react with H_2O_2; these compounds include α-ketobutyrate, which is not an energy substrate; (2) lactate, which can be used instead of pyruvate as a neuronal energy substrate (Schurr et al., 1988), was ineffective in protecting neurons against H_2O_2-induced toxicity; and (3) the neuroprotective effect of the different α-ketoacids against H_2O_2 toxicity was closely correlated with their ability to scavenge H_2O_2.

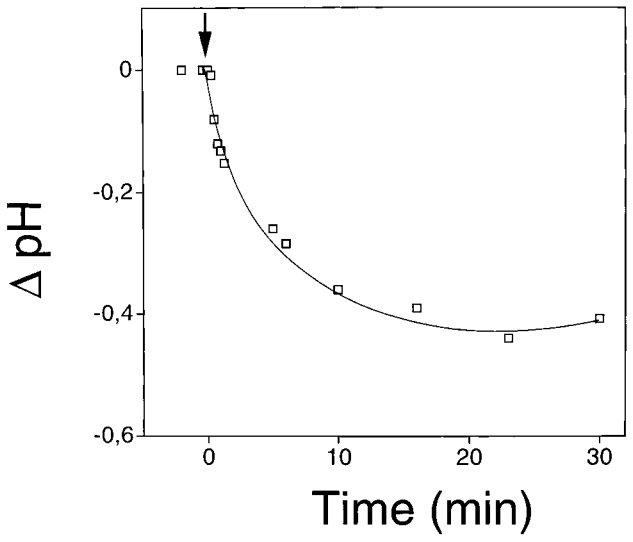

Figure 7. Cytosolic acidification by 10 mM sodium pyruvate. Cultured striatal neurons, previously loaded with carboxy-SNARF-1, were perfused for 30 min with 10 mM sodium pyruvate (*arrow*) in Krebs' bicarbonate buffer at a constant extracellular pH of 7.4. The exposure to pyruvate resulted in a long-lasting decrease of the 580:640 nm fluorescence ratio, determined as described in Materials and Methods. After pyruvate removal, the ratio increased, returning to its resting value by the end of a 25 min washout (data not shown). Each *point* is the mean from 14 cells. Two other independent experiments gave similar results.

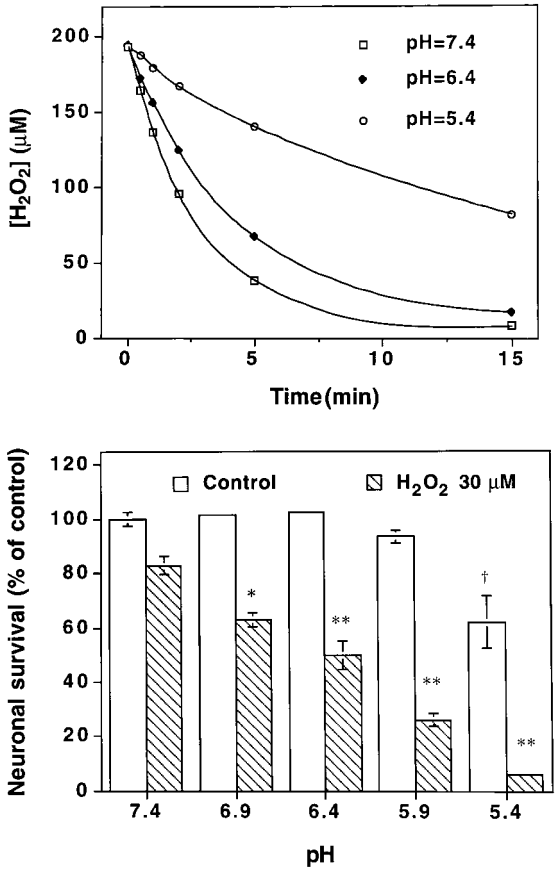

Figure 8. Influence of pH on the neuroprotective effect of pyruvate. *Top*, Kinetics of H_2O_2 degradation by pyruvate in acid solutions. Pyruvate (2 mM) and H_2O_2 (200 μM) were incubated at 37°C in the absence of cells in HEPES-buffered salt solutions adjusted to different pH for increasing times. The residual concentrations of H_2O_2 were determined as described in Materials and Methods. Data are the mean ± SEM of three independent experiments, each performed in triplicate. The error bars are not visible, because they are smaller than the symbols. *Bottom*, Neurotoxic effects of H_2O_2 in acid solutions. Cultured neurons were preincubated for 15 min and then incubated for 30 min with or without 30 μM H_2O_2 in HEPES-buffered salt solutions adjusted to different pH. Neuronal survival was estimated 24 hr later. Results are expressed as the percentage of living neurons compared with control cultures incubated at pH 7.4 in the absence of H_2O_2. Data are the mean ± SEM of three independent experiments, each performed in triplicate. $^{\dagger}p < 0.01$; significantly different from the control value; $^{*}p < 0.05$; $^{**}p < 0.01$; significantly different from the value obtained in the presence of H_2O_2 at pH 7.4 (ANOVA followed by Dunnett's test).

As estimated by microdialysis in the ischemic striatum, the concentration of H_2O_2 can reach as much as 100 μM during the reperfusion phase (Hyslop et al., 1995). In this situation, H_2O_2 is believed to be produced by cells located in the ischemic brain area and released into the extracellular space. Our results indicate that striatal neurons exposed to menadione also produce and release H_2O_2 in the incubating medium, and that pyruvate protects striatal neurons against the quinone-induced toxicity. Supporting this statement and showing that released H_2O_2 contributes to the toxic effect of menadione, the addition of catalase into the incubating medium partially protected the striatal neurons from the menadione-induced neurotoxicity. The higher neuroprotective effect of pyruvate can be related to its capacity to enter the cells and therefore to scavenge intracellular H_2O_2. Such beneficial effects of pyruvate have already been observed in human breast carcinoma cells and in the LLC-PK_1 cells derived from the renal tubular epithelium (Nath et al., 1995).

When striatal neurons are exposed to menadione, H_2O_2 is produced in the vicinity of free iron sources such as microsomes and mitochondria (Nath et al., 1995). Some toxic hydroxyl radicals could thus be formed before the scavenging action of pyruvate toward H_2O_2. This could explain why the protective effect of pyruvate against menadione-induced toxicity was less pronounced than that observed under the exposure of striatal neurons to exogenous H_2O_2.

Our study also indicates that at high concentration, pyruvate induces an intracellular acidification, which probably interferes with its neuroprotective effect. The cytosolic acidification of striatal neurons induced by 10 mM pyruvate may result from the H^+ cotransport across the plasma membrane by the specific H^+-monocarboxylate cotransporter (Nedergaard and Goldman, 1993; Poole and Halestrap, 1993) and to a lesser extent from the diffusion of undissociated pyruvic acid (Bakker and Van Dam, 1974). Furthermore, intracellular acidification enhanced the neurotoxic effect of H_2O_2 and reduced the rate of H_2O_2 scavenging by pyruvate, as already reported by Melzer and Schmidt (1988). The reduction of intracellular pH is known to induce the release of active iron from ferritin (Funk et al., 1985; Braughler and Hall, 1989), a process that leads to an enhanced production of $OH^{\bullet}$ (Siesjö et al., 1985; Rehncrona et al., 1989). In addition, small reductions in pHi can inhibit metabolic enzymes (Busa, 1986). All of these events could contribute to an enhanced neurotoxic effect of H_2O_2 intervening under the intracellular acidification induced by 10 mM pyruvate. The optimal neuroprotective concentration of pyruvate should be reached when its H_2O_2-scavenging capacity exceeds its adverse effect linked to the cytosolic acidification. When striatal neurons were exposed to menadione, this optimal neuroprotective concentration of pyruvate was ~1 mM.

According to O'Donnell-Tormey et al. (1987), pyruvate is the sole α-ketoacid that is secreted, its extracellular concentration reaching almost its intracellular concentration. Therefore, in pathological situations such as brain ischemia or trauma, endogenously produced pyruvate could be considered an extracellular antioxidant. Indeed, under these circumstances, released H_2O_2, which exerts its toxic effect through a paracrine process, could be scavenged by external pyruvate. We have recently demonstrated that astrocytes strongly protect neurons against external H_2O_2 by degrading the oxidant and that catalase is the main astrocytic enzyme activity responsible for this neuroprotective effect. If H_2O_2 scavenging by external pyruvate occurs *in vivo*, the beneficial role of astrocytes should depend not only on their hydrogen peroxidase activity but also on their glycolytic activity and their capacity to release pyruvate. Pellerin and Magistretti (1994) have demonstrated that glutamate, which is largely released under ischemia, can stimulate glycolysis in astrocytes and can increase lactate and pyruvate release from these cells. Therefore, pyruvate, which originates from astrocytes and which has a release process that is submitted to regulation, might contribute to neuronal protection.

Glucose metabolism impairment has been reported to occur in neurodegenerative disorders such as Alzheimer's and Huntington's diseases or amyotrophic lateral sclerosis (Beal, 1992). Therefore, the decline in pyruvate levels that may occur in such pathological situations should result not only in a deficit of energy metabolism but also in a reduced antioxidant effect of this agent. This reduced antioxidant state is likely to contribute to a higher neuronal vulnerability to reactive oxygen species and consequently to neuronal death.

Unlike exogenous catalase, pyruvate and other α-ketoacids can cross the blood–brain barrier (Oldendorf, 1973; Conn et al., 1983). Therefore, our study suggests that pyruvate and other α-ketoacids could be of therapeutic value in pathological situations, such as ischemia-reperfusion or trauma, in which acute production of H_2O_2 is believed to play a critical role. Indeed, intravenous infusions of pyruvate leading to millimolar plasma concentrations of this α-ketoacid are tolerated without apparent adverse effect in humans (Dijkstra et al., 1984).

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