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Prevention of Intracellular Oxidative Stress to Lens by Pyruvate and Its Ester

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Pyruvate is a well-known scavenger of hydrogen peroxide (H_2O_2). In addition, it scavenges superoxide radical ($O_2^{\bullet-}$). However, evidence on its intracellular antioxidant function is meager at present. Hence, we have examined the effectiveness of this metabolite and its ethyl ester against intracellular oxidative damage to the lens under organ culture. Menadione, a redoxcycling quinone, was used to generate the reactive oxygen species (ROS). It was found to inhibit lens metabolism as evidenced by a decrease of ATP. Additionally, tissue oxidation was apparent by loss of glutathione (GSH), and increase in the level of oxidized glutathione (GSSG), coupled with increase of the urea soluble proteins (water insoluble). The overall physiological damage was apparent by the inhibition of the Na^+-K^+-ATPase dependent cation pump, as evidenced by a decreased rubidium transport. These deleterious effects were attenuated by pyruvate and ethyl-pyruvate. The later was found to be more effective.

Keywords: Oxygen radicals, cataract, eye lens, menadione, pyruvate, pyruvate-ester

INTRODUCTION

Tissue damage by reactive species of oxygen (ROS) is known to be involved in the genesis of several age dependent pathologies such as arteriosclerosis, cancer, emphysema, Alzheimer's disease, macular degeneration and cataracts.[1–6] This study pertains to the protection against oxidative stress to the ocular lens and consequent cataract formation. It is believed that the long latent period required for cataract development is due to the retarding effects of the various enzymatic ROS scavengers such as superoxide dismutase (SOD), glutathione peroxidase (GSHPX) and catalase. But, because of their large size, their role in preventing oxidation at sites not easily accessible, such as in the buried protein and lipid structures, becomes less significant. The prevention of such site-specific oxidation, however, can be accomplished by freely diffusible antioxidants, such as ascorbate in the hydrophilic and tocopherols in the hydrophobic sites.[7–9] Nevertheless, their availability in human and other primates can become self-limiting due to poor dietary intake as well as the deterioration of the blood aqueous transport barrier. In addition, their quinoids can have some toxicity. Studies

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were hence considered necessary to investigate the possibility of preventing oxidative stress in lens by certain small molecular weight endogenous compounds. We have hypothesized that pyruvate may be one such compound. Studies on its antioxidant function are based on its property of scavenging H_2O_2.[10] It also prolongs the life of certain bacterial strains cultured aerobically.[11] Subsequently, we have demonstrated that it can also protect the lens under culture against peroxide damage.[12–14] However, addition of catalase to the culture medium is also effective.[4] Hence, these results fail to provide any direct evidence of the usefulness of this compound as an intracellular antioxidant. The above findings with lens culture when considered along with that of catalase could be explained on the basis that the pyruvate effect is due to the rapid decomposition of the medium peroxide, prior to its penetration into the cells. The objective of these studies was hence to investigate more specifically if pyruvate can prevent against intracellular oxidative stress. With the possibility that it may not penetrate the cells adequately because of its charge, studies were extended with pyruvate-ester (ethyl pyruvate). The results demonstrate that pyruvate indeed can function as an intracellular antioxidant, especially if used in the ester form. Menadione was used to induce intracellular oxidation.

MATERIAL AND METHODS

Most non-radioactive chemicals were obtained from Sigma Chemical Co, St. Louis, MO 63178, USA. Radioactive rubidium chloride was obtained from New England nuclear Company, Boston, MA.

All experiments were carried on lenses obtained from Sprague-Dawley rats (150±15g), used in accordance with the ARVO guidelines. They were killed by exposure to CO_2. The eyes were then enucleated, rinsed with physiological saline and intact lenses dissected out atraumatically. They were then organ cultured contralaterally for 16–18 hours by gently transferring them to culture dishes (35×10mm) containing 4 ml of Tyrode medium maintained at 37°C, in an incubator gassed with 95% air: 5% CO_2, in the absence or presence of 40μM menadione. The physiological damage induced by the quinone, was assessed in terms of an inhibition of the lens cation pump activity as evidenced by the decreased transport of $^{86}Rb^+$ ions against a concentration gradient.[6] The extent of oxidative damage was measured in terms of lens GSH, GSSG and the content of urea soluble proteins. To determine GSH and GSSG, the individual lenses were homogenized in 1 ml of 5% trichloroacetic acid. The homogenate was centrifuged to obtain a clear supernatant. For GSH determination, 0.1ml of this supernatant was then mixed with 0.8ml of 0.3 M Na_2HPO_4 and 0.1ml of DTNB reagent and quantitation done spectrophotometrically at 410 nm (14). For GSSG determination, 0.1ml of the above acid extract was mixed with 0.8ml of 0.6 M phosphate buffer containing 30 mM disodium-EDTA, pH 7.4, 0.1ml of 0.33mM NADPH and 5μl (2–5 units) of glutathione reductase (sigma G4759). The decrease in absorbance at 340 nm was determined by reference to a cuvette containing the blank reaction mixture, wherein glutathione reductase was omitted. A reference standard was run simultaneously. The overall metabolic effect was measured in terms of the levels of ATP. For this purpose, individual lenses were homogenized in 1ml of deionized water and a soluble extract obtained by centrifugation in the cold. A luciferin/luciferase reagent was prepared by adding 5 ml of distilled H_2O to the sigma vial (FLE-50) containing dehydrated firefly lanterns and buffer components. 200μl of this reagent was then mixed with 50μl of the above lens extract in a luminometer and the luminescence produced recorded. Standards were run simultaneously.[14]

The water insoluble precipitate obtained in the above experiment was washed 3 times by mixing and centrifugation with 1 ml of distilled water. The residue was then dissolved by heating it at 37°C for 30 minutes in 0.3 ml of 7 M urea

containing 50 mM Tris, pH 7.4. The supernatant thus obtained was designated as urea soluble proteins and its content determined using Biorad's protein reagent.[14]

In order to determine the efficacy of pyruvate and pyruvate-ester against menadione-mediated intracellular oxidative damage, contralateral lenses were used, where one lens was incubated with menadione and the other lens was incubated with menadione plus pyruvate or its ester. In view of the continued production of $O_2^{\cdot -}$ by the redox cycling of menadione, pyruvate and its ester were used at 5mM. Lower levels were less effective. The post incubation lenses were analyzed for rubidium uptake and the levels of GSH, GSSG, ATP and urea soluble proteins as described above.

RESULTS

Figure 1 summarizes the distribution ratios of rubidium between lens water and medium (CL/CM) attained under different conditions following an overnight incubation. The ratio obtained in the basal medium (control) was 47 ± 2. With the medium potassium being 2.7 mM, and with rubidium acting as a surrogate ion, this ratio represents an intracellular lens

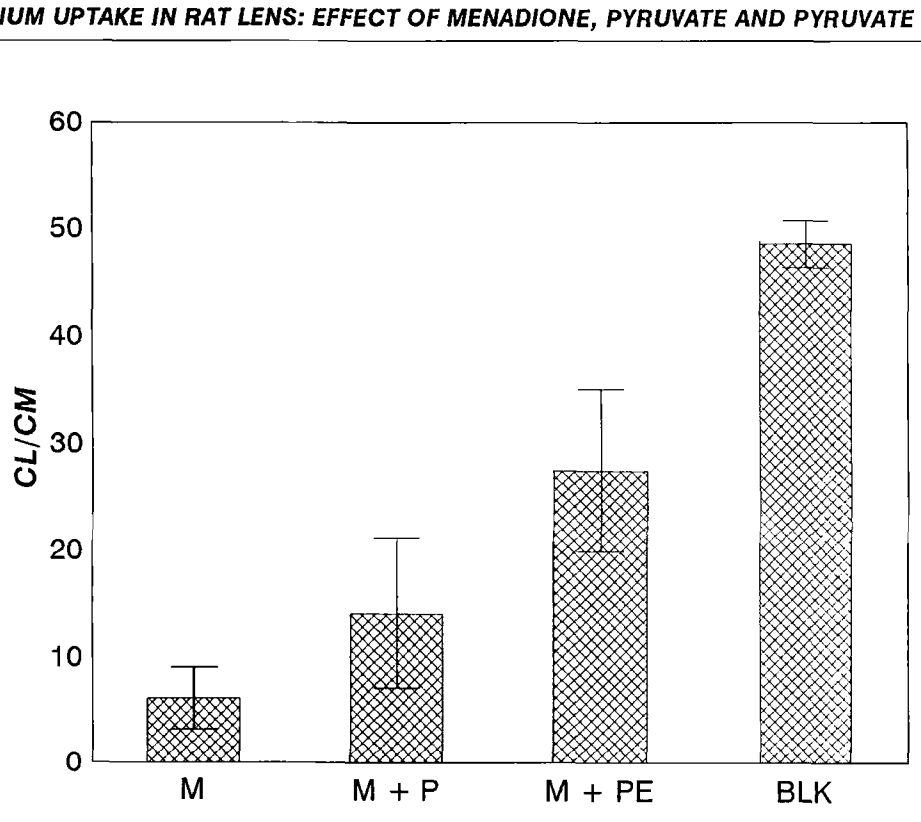

FIGURE 1 Active transport of rubidium ions in the lens: Freshly dissected rat lenses were incubated for 16–18 hours in Tyrode medium pulsed with ^{86}RBCl and containing 40 μM menadione, in the absence or presence of 5 mM pyruvate or its ethyl ester. Following incubation, the distribution ratio (CL/CM) was determined by dividing the radioactivity present in the lens (CL) with the radioactivity present in an equivalent volume of the medium (CM). M = Menadione, M + P = Mendione + Pyruvate, M + PE = Menadione + pyruvate ester, BLK = Blank control (incubated in basal medium). At least 6 experiments were conducted in each case. The values are mean ± standard deviation.

potassium to be ≈ 120 mM. Hence, the lenses maintain themselves in good physiology under the basal incubation conditions. In the presence of menadione, this ratio decreased to 6±3, demonstrating a substantial physiological damage to the cation pump. This could be attributed to a direct inhibition of the Na^+-K^+-ATPase, as well as to a generalized toxic effect of ROS. The metabolic damage was apparent by a decrease in the ATP levels (Table I). The content of this nucleotide in the basal control lenses was ≈ 2,000 nmoles/g wet weight. In the presence of menadione, it decreased to about 1/10th of the basal value (≈ 200 nmoles/g). That oxidation was a significant contributing factor in menadione-induced damage was apparent by a loss of GSH, the main antioxidant reserve of the lens. Its content in the basal control was ≈ 4 μmole/g, falling to 1 μmole in the presence of menadione (Table I).

After establishing the above parameters for measuring menadione damage, experiments were conducted to determine the possible preventive effects of pyruvate and its ester. As summarized in Table I, pyruvate remained ineffective in preventing glutathione loss, even though only micromolar levels of menadione were used. This relative ineffectiveness is probably due to its poor penetration in to the cells, as well as to its rapid utilization in several other competing reactions. This is apparent from a small but significant increase in the ATP content (Table I). It also offered significant protection against the cation pump damage as apparent by an increase in the distribution ratio (Figure 1). In view of the fact that the ester forms of carboxylic acids penetrate cell membranes better than the free acids, subsequent studies were carried out with pyruvate-ester. Such esters once inside the cell, are hydrolyzed by the esterases, liberating the parent acids. As summarized in Figure 1, the distribution ratio of rubidium was now much higher (≈ 27) in comparison to that observed in presence of menadione (≈ 6) as well as to that observed in presence of menadione plus pyruvate (13). The ATP level was also better maintained in the presence of the pyruvate ester. In addition, its antioxidant property is more definitively demonstrated, by at least a 2 fold elevation in the level of GSH in comparison to the menadione group.

The protective effect of pyruvate-ester was also apparent by the decrease in the levels of GSSG in comparison to the menadione group. The basal lens contained only minute amounts of GSSG (0.2mM). It increased to 0.645mM on incubation with menadione. In the lenses incubated with pyruvate and the pyruvate-ester along with menadione, GSSG increase was minimized. Overall, the results demonstrate for the first time that despite several competing metabolic reactions, elevation of pyruvate in the cytosol offers a viable means of preventing intracellular oxidative stress and the development of related pathologies. Addition of 5mM ethanol, the hydrolysis product of pyruvate-ester to menadione

TABLE I

	ATP nmole/gm	GSSG μmol/gm	GSH μmol/gm	Water insoluble (urea soluble) protein mg/g lens
(A) Menadione (40μM)	222±95	0.65±.034	1.04±0.13	55.83±5.9
(B) Pyruvate 5mM	533±289	0.332±.07	0.97±0.3	47.83±12.7
(C) 5mM Pyruvate ester	1216±388	0.388±.034	2.10±0.58	41.05±1.6
(D) Blank control	2147±274	0.201±0.02	3.68±1.0	36.01±8.7

Oxidative stress on lens by menadione. Prevention by pyruvate and its ethyl ester. The number of lenses analyzed in each case was 6. The mean weight of the lenses was 30±2 mg. The results are expressed as Mean±Standard Deviation. P values are as follows:

ATP	Between A&D ≤ .001, A&B ≤ .001, A&C < .001
urea soluble protein	Between A&D ≤ .005, A&B ≤ 0.1, A&C = .05
GSH	Between A&D ≤ .001, A&B < *N.S.*, A&C < .001.
GSSG	Between A&D ≤ 0.003, A&B ≤ 0.025, A&C ≤ 0.025

Determinations of ATP, GSH, GSSG, and proteins were done as described in methods.

containing medium was ineffective, demonstrating that the protective effect of the ester is primarily due to the pyruvate produced.

DISCUSSION

Oxy-radical induced damage to the tissues has been implicated in the development of several acute and chronic diseases.[1–6] Such damage does take place despite the presence of several enzymatic defenses. This may be due to the generation of active oxygen species in quantities beyond the capacity of the defense enzymes. More likely, it may be due to an inaccessibility of the enzymes and their cofactors (if required) at appropriate sites in the macromolecular organization. Prevention of oxidative stress by small molecular weight substances such as ascorbate and pyruvate may therefore become desirable in situations giving rise to excessive oxidative stress. In this communication, we have studied the effect of pyruvate and its ethyl ester. Previous studies with pyruvate were done in lens organ culture systems where an oxidant (H_2O_2 or xanthine + xanthine oxidase) was added to the culture medium in the absence and presence of pyruvate. Because of the rapid scavenging of these oxidants by pyruvate, the systems used above might demonstrate the effectiveness of pyruvate primarily against the extracellular membrane oxidation.[12–14] Its significance in the intracellular compartment, especially in the presence of an oxidant that undergoes redox cycling could not be ascertained. Hence, it was considered necessary to study further the usefulness of this substance in preventing intracellular oxidation. The oxidant used was menadione. Addition of even micromolar quantities of this quinone was found to cause changes at millimolar levels due to oxidative stress. The prevention of such damage therefore demonstrates more convincingly the usefulness of pyruvate as a possible physiological antioxidant as well as providing metabolic support to the lens under various stress.[13] It is further interesting to note that the esterified pyruvate is more effective. Hence, studies with other esters of pyruvate are likely to help in the development of more effective antioxidants suitable for therapeutic applications. Such studies are in progress.

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