Published in final edited form as:

Mol Cell Biochem. 2010 October ; 343(1-2): 101–105. doi:10.1007/s11010-010-0503-9.

Inhibition of glycolysis in the retina by oxidative stress: prevention by pyruvate

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Abstract

Intraocular generation of reactive oxygen species (ROS) with consequent oxidative stress has been shown to be a significant factor in the pathogenesis of many vision-impairing diseases such as cataracts and retinal degenerations. Previous studies have shown that pyruvate can inhibit such oxidative stress. This is attributable to its property of scavenging various ROS and consequently inhibiting many of the apparent toxic reactions such as lipid peroxidation and loss of tissue thiols. It is hence expected that ROS will have an adverse effect on tissue metabolism also. The present investigations were hence undertaken to study the possibility that while scavenging ROS, the compound could be effective also in preventing the inhibition of tissue metabolism as well. Since glycolysis constitutes the major bioenergetic source of the retina, the objective of the present studies was to ascertain if the effects of pyruvate are indeed reflected in the maintenance of this pathway even when the tissue is exposed to ROS. This hypothesis was examined by incubating retinal explants in ROS-generating medium in the absence and presence of pyruvate and measuring $^3\text{H}_2\text{O}$ generated from 5- ^3H glucose. In addition, the lactate generated was also measured. As hypothesized, ROS-induced inhibition of glycolysis indexed by the decrease in $^3\text{H}_2\text{O}$ as well as lactate formation was significantly prevented by pyruvate. This effect was also reflected by the elevation of NAD/NADH ratio, a major pacemaker of glycolysis.

Keywords

Glycolysis in retina; Retinal oxidative stress; Pyruvate; Enolase action; Formation of tritiated water; NAD/NADH; Lactate/pyruvate

Introduction

Excessive generation of reactive oxygen species (ROS) and the consequent induction of oxidative stress are considered to be significant factors in the pathogenesis of several ocular

diseases including diabetic retinopathy, age-related macular degeneration, glaucomatous neurodegeneration, uveoretinitis, and cataracts [1–5]. It is generally believed that in most tissues about 2–4% of the respired oxygen undergoes monovalent reduction generating O_2^- during electron transport through the cytochromes [6]. This free radical and its derivatives viz., H_2O_2 and the OH^- are effectively scavenged by endogenous enzymatic scavengers such as catalase, superoxide dismutase and glutathione peroxidase. They are also scavenged non-enzymatically (though less effectively) by several compounds such as ascorbate, tocopherol, glutathione and the pyridine nucleotides. However, ROS generation can become excessive with aging, diabetes, inflammation, nutritional deficiencies and exposure to actinic radiation including sunlight. Hence, the endogenous defense mechanisms can become inadequate to detoxify the ROS with the consequence of aberrant oxidation of several susceptible cellular components, such as the non-enzymatic and enzymatic protein sulphhydryls, glutathione, lipids, sugars, and the nucleic acids, with adverse effects on cellular metabolism and eventual cell death. The retina could be especially susceptible to such oxidative damage due to its higher oxygen consumption as compared to most other body tissues [7]. The consequent high flux of oxygen in this tissue through the mitochondrial respiratory chain raises the possibility of increased production of oxyradicals. There is also a simultaneous increase in extra-mitochondrial production of ROS in the cytosol, leading to oxidative deactivation of many enzymes involved in regulating glycolysis- glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and pyruvate kinase being particularly sensitive [8,9]. Since the tissue is bioenergetically heavily dependent on glycolysis for its function of phototransduction [10, 11] and processing of visual information, the objective of the present investigations was to determine the possible preventive effect of pyruvate against such metabolic damage. The protective effect of this compound against oxidative damage to the retina was hypothesized on the basis of earlier studies showing it to be a highly effective scavenger of most of the ROS [12,13]. In addition, it is hypothesized that it could facilitate glycolysis by the recycling of NAD^+ required for the continued activity of the glycolytic pathway. We have examined these hypotheses by determining the status of glycolysis in retinas incubated with oxyradical species generated enzymatically by xanthine-xanthine oxidase reaction. The extent of glycolysis was determined by incubating the tissue in medium pulsed with 5- 3H glucose and determining the amount of 3H_2O generated. This was assessed further by measuring lactate production by the tissue incubated with and without pyruvate as well as determining the $NAD^+/NADH$ ratio.

Materials and methods

C57Bl/6 mice (30–35 g), obtained from Harlan Laboratories, were used according to the guidelines of the Institutional Animal Care and Use Committee and Association for Research in Vision and Ophthalmology. All non-radioactive chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). 5- 3H glucose was obtained from Perkin Elmer (Cat # NET531001MC, 1 mCi, Boston, MA). Anion exchange resin (AG1X8 OH form, 200–400 mesh, Catalogue # 143-2446) and boronate affinity gel (Affi-Gel, Catalogue # 153-6103) were obtained from Bio-Rad Laboratories (Hercules, CA).

Mice were euthanized and the neural retina obtained as follows: a 3-mm corneal incision (close to the limbus) was made and the intact neural retina extruded through the incision by application of pressure behind the globe. The retinas were individually incubated in 0.25 ml medium 199 (containing 5.5 mM glucose and 0.5 mM xanthine (XA)). The medium also contained 5- 3H glucose (10 μ l/5 ml of medium). The specific activity was 250–300 CPM/nanomole of glucose; 10 μ l of xanthine oxidase (XO, Sigma X 2252) containing 0.07 U of the enzyme was then added. The amounts of XA–XO were adjusted so that the level of H_2O_2 generated was ~0.3 mM by 1 h. Parallel incubations were conducted with the addition of sodium pyruvate (10 mM). Basal controls were run without xanthine oxidase in the absence and presence of

pyruvate. Incubations were carried out for 3.5 h at 37°C in a humidified incubator gassed with 5% CO₂. The contents of ³H₂O, lactate, and pyruvate were measured as follows:

Determination of ³H₂O

50–100 µl of the post-incubation medium was loaded on an anion exchange hydroxide column (1 cm × 0.7 cm) piggybacked on a boronate affinity column (3 cm × 0.7 cm). The columns were eluted with 6–10 ml of dH₂O. Glucose (including the radioactive glucose), charged metabolites of glycolysis, and lactate remained bound and retained on the hydroxide column. Any free glucose not bound on the above column and hence leaking through it gets complexed and bound to the boronate affinity column. The radioactivity remaining in the eluate, representing 3H₂O, was quantified by liquid scintillation counting. The amount generated was expressed as per milligram protein determined colorimetrically by Bradford's method by coaming an aliquot of the retinal homogenate prepared by sonication in 0.5 ml dH₂O with Coomassie Blue G-250 based BioRad protein reagent.

NAD⁺/NADH ratio

Following incubation of the neural retina as described above, the tissue was immediately sonicated at 4°C in its own post-incubation medium and the total protein was determined as above. The homogenate was centrifuged and the contents of lactate and pyruvate were determined in the supernatant.

Measurement of lactate

Lactate was determined enzymatically by noting the increase in the absorption at 340 nm due to the formation of NADH during its oxidation to pyruvate in the presence of lactate dehydrogenase [14]. Briefly, aliquots of the supernatant were added to a reaction mixture containing NAD (0.08 mM) and lactate dehydrogenase (10 U) in glycine (1M)-hydrazine (0.4M) buffer, pH 9.5. A reagent blank was run simultaneously using the unincubated medium. The cuvettes were maintained at 37°C and readings were recorded for approximately 15 min to ensure the completion of the reaction.

Measurement of pyruvate

Pyruvate concentration was determined enzymatically by mixing the sample with NADH and LDH reagent and monitoring spectrophotometrically the decrease in absorption (at 340 nm) due to the NADH-dependent reduction of pyruvate to lactate by LDH. Briefly, the assay was done as follows: a reagent mixture consisting of 0.09 mM NADH in 0.05M phosphate buffer, pH 7.4, and LDH (40 units) was prepared and the initial absorption (OD₁) noted; 10 µl of the tissue extract was then added to the reaction mixture (0.4 ml) and OD₂ was read after 10 min. Pyruvate concentration, represented by the difference in absorption (OD₁–OD₂), was calculated with reference to the extinction coefficient of NADH.

Pyruvate and lactate levels so determined were used to determine the NAD⁺/NADH ratios based on the equilibrium constant of the reaction using the following equation:

$$\frac{[\text{NAD}^+]}{[\text{NADH}]} = \frac{[\text{Pyruvate}][\text{H}^+]}{[\text{Lactate}]} \times \frac{1}{K}$$

The *K* value calculated by Williamson, Lund, and Krebs [15] using the above equation is 1.1 × 10⁻¹¹ M. This also tallies with the value we obtained on the basis of Δ*G*^o = -25.1 kJ/mole.

Results

Results on the production of $^3\text{H}_2\text{O}$ by the retina incubated as described above are summarized in Fig. 1. As may be noted, the amount of $^3\text{H}_2\text{O}$ produced by the retina on incubation in the basal medium without XO was ~ 1.63 $\mu\text{moles/mg protein}$, in the absence as well as in the presence of pyruvate. The addition of XO to the medium was significantly inhibitory, the amount produced in this case being only ~ 0.75 $\mu\text{moles/mg protein}$. This inhibition is attributable to the metabolic damage to the tissue induced by the superoxide and its derivatives produced in the reaction mixture. As indicated, addition of pyruvate (10 mM) to the medium was highly effective in abolishing this inhibition, the amount of $^3\text{H}_2\text{O}$ in this case being ~ 1.7 $\mu\text{moles/mg protein}$, close to the control value.

The protective effect of pyruvate was also apparent by the contents of lactate and pyruvate in the tissue and incubation medium. As shown in Table 1, the amount of lactate produced per milligram of protein was 8.5 and 9.4 mM in the control groups incubated in the basal medium in the absence and presence of pyruvate, respectively. It decreased to 3.5 mM in the presence of ROS. This decrease was substantially prevented when the retina was incubated with pyruvate despite the presence of xanthine and xanthine oxidase in the medium, the value in this case being ~ 6.1 mM/mg protein. The concentration of pyruvate also decreased from ~ 0.9 mM in the control group, incubated in the basal medium without exogenously added pyruvate, to ~ 0.05 mM in the presence of ROS. As expected, the addition of pyruvate to the ROS generating medium increased its level to 5.6 mM/mg protein, altering the nucleotide levels in such a way as to favor glycolysis.

As shown in Table 1, the NAD^+/NADH ratio decreased from ~ 616 in the basal controls [16], incubated without ROS and pyruvate, to ~ 111 in the presence of ROS. However, the ratio was substantially elevated by the addition of pyruvate, the value in this case being 6700, which was close to that obtained in controls incubated with pyruvate without XO.

As indicated, the lactate/pyruvate ratio was also adversely affected by oxidative stress. As shown in Table 1, the ratio increased from ~ 10 in the controls to ~ 70 in the presence of ROS. This was completely prevented by exogenous pyruvate, the ratio in this case being ~ 1 .

Pyruvate was hence found to be substantially effective in preventing ROS-induced inhibition of glycolysis in the retina.

Discussion

ROS-induced oxidative stress has been implicated in the pathogenesis of several vision-impairing eye diseases such as cataracts, glaucoma, Eales' disease, age-related macular degeneration, diabetic retinopathy, and chorioretinitis, etc. These species, especially the $\text{OH}^\cdot$, cause highly deleterious effects on the tissue physiology through several aberrant reactions such as oxidative modifications of protein and non-protein sulphhydryls. The oxidation of susceptible thiols at the catalytic sites, in several enzymatic proteins [8,9], is known to lead to their inactivation and consequent metabolic inhibition leading to a decrease in the generation of ATP, required for conducting active transport and supporting various biosynthetic and repair processes. Such metabolic inhibition is known to occur in many conditions such as diabetes known to be associated with the decrease in the respiratory quotient (RQ). Hence, excess ROS generation in these diseases can be attributed to the lowered utilization of glucose with consequent increase in the availability of unused oxygen [17,18].

The primary objective of these studies was therefore to determine whether pyruvate, an endogenous alpha keto acid metabolite, could prevent oxidative-stress-induced inhibition of glycolysis in the retina, this pathway being the major mode of glucose utilization and primary

source of energy in the neural tissue [10,11]. The reactions of pyruvate with all the ROS, including the hydroxyl radical, are known to be highly favorable thermodynamically. Its ability to scavenge hydrogen peroxide was shown as early as 1900s by Fenton [12] and Holleman [13]. The rate constant of its reaction with the hydroxyl radical in aqueous solution has been most recently determined to be 5×10^8 to $9 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$, close to the diffusion limit [19]. In addition to its effectiveness in scavenging oxygen radicals in pure chemical reactions as referred above, its physiological effectiveness in preventing oxidative stress was first demonstrated by Maiweg, as apparent by its effectiveness in preventing microbial death induced by exposure to excessive oxygen [20]. That it might be effective in preventing eye diseases involving oxidative stress as a causative factor was first demonstrated by us using the lens as a model eye tissue [21]. The physiological effectiveness against ROS damage was convincingly demonstrated by its ability to prevent the inhibition of active transport in the tissue as well as maintaining the levels of ATP and glutathione. It was also found to be effective in preventing actual cataract formation induced by sodium selenite as well as diabetes [22, 23]. Our more recent studies suggest that it could be effective in preventing oxidative stress induced damage to the retina also [24]. We now consider that the observed protective effect of pyruvate is exerted not only through its potent oxyradical scavenging properties but also through its ability to stimulate tissue metabolism through acceleration of glycolysis. As demonstrated by the results described here, exposure of the retina to reactive oxygen led to a highly significant depression of glycolysis, indexed by the decrease in the generation of $^3\text{H}_2\text{O}$ from $5\text{-}^3\text{H}$ glucose (Fig. 1). Lactate production (Table 1) was also significantly diminished. Pyruvate was highly effective in antagonizing these effects. The mechanism by which pyruvate helps in the maintenance of tissue glycolysis could be attributed to the inhibition of oxidative inactivation of $-\text{SH}$ containing enzymes such as GAPDH and pyruvate kinase [8,9,25,26]. The stimulatory effect on glycolysis is explained also on the basis of the continued regeneration of NAD^+ , produced during the reduction of pyruvate to lactate by LDH, and consequent stimulation of the oxidation of glyceraldehyde-3-phosphate to 1,3 diphosphoglycerate by GAPDH dependent on NAD^+ availability. This is proven by the maintenance of NAD^+/NADH ratios at levels significantly higher in the presence of pyruvate as compared to that in its absence, in the controls as well as in those incubated with ROS (Table 1). Hence, the overall protective effect of pyruvate is proposed to be exerted through its dual mode of action as a scavenger of ROS as well as its action as a metabolic stimulant, both effects being particularly important in case of the retinal tissue known to be highly susceptible to oxidative stress due to its high rate of oxygen utilization. This high level of oxygen utilization increases the chance of ROS generation. Additionally the high susceptibility of the retina to oxidative damage could also be attributed to the high abundance of polyunsaturated fatty acids, providing unsaturated sites for radical attack and generation of toxic aldehydes. The ROS generation in this tissue is also augmented by rhodopsin and other photosensitizers that can photochemically generate ROS during photopic vision involving constant exposure of the retina to light [27]. Further studies on the induction of retinal cell damage by oxidative stress and its possible prevention by pyruvate are under progress.

Acknowledgments

Acknowledgments The authors are thankful for the financial support of NEI, NIH and Research to Prevent Blindness Inc, New York.

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Fig. 1.

Determination of glycolysis by measurement of $^3\text{H}_2\text{O}$. Retinas isolated from C57Bl/6 mice were incubated with 5- ^3H glucose in medium 199 with and without XO and pyruvate as described in the text. $^3\text{H}_2\text{O}$ produced was determined following its chromatographic separation. The values represent mean $\pm$ SD; $n = 6$ in each group. The amount of $^3\text{H}_2\text{O}$ generated by the tissue in presence of ROS + pyruvate is significantly higher as compared to that with ROS alone, $P < 0.001$

Table 1Modulation of NAD⁺/NADH ratio in the retina by pyruvate

	Lactate (L) (mM/mg protein)	Pyruvate (P) (mM/mg protein)	Lactate/pyruvate	NAD ⁺ /NADH
Control	8.5 ± 1.8	0.89 ± 0.15	10 ± 3.5	616 ± 140
Control + P	9.4 ± 1.5	8.2 ± 4	1 ± 0.3	8220 ± 2300
XA + XO	3.5 ± 0.6*	0.05 ± 0.03*	70 ± 22*	111 ± 35*
XA + XO + P	6.1 ± 1.5**	5.6 ± 2.1**	1 ± 0.1**	6715 ± 1250**

The values represent mean ± SD; *n* = 8 in each group. Notable is the decrease in the NAD⁺/NADH ratio by oxidative stress, pyruvate being preventive.

* *P* < 0.001 for difference from controls

** *P* < 0.001 for difference from XA + XO