References


Nicotinamide N-Oxide Reductase Activity in Bovine and Rabbit Eyes

Shigeaki Shimada,* Hiromu Mishima,* Shigeyuki Kiramuratu, and Kiyoshi Tarsumif

The nicotinamide N-oxide reductase activity of a variety of ocular tissues was investigated. The 9,000g supernatant of ciliary body, retinal pigment epithelium-choroid, iris, retina and cornea, but not lens, exhibited reductase activity under anaerobic conditions when supplemented with 2-hydroxypyrimidine, an electron donor of aldehyde oxidase. Among these tissues, the highest activity was observed with ciliary body. When the 9,000g supernatant of ciliary body was fractionated, the 2-hydroxypyrimidine-linked reductase activity was mainly associated with the cytosolic fraction and was markedly inhibited by menadione, an inhibitor of aldehyde oxidase. Similarly, in the presence of 2-hydroxypyrimidine, the cytosolic fraction of rabbit ciliary body exhibited nicotinamide N-oxide reductase activity which was susceptible to inhibition by menadione. These facts strongly suggest that aldehyde oxidase present in mammalian eyes is involved in the reduction of nicotinamide N-oxide to nicotinamide. Invest Ophthalmol Vis Sci 28:1204–1206, 1987

Nicotinamide N-oxide has been recognized as an in vivo metabolite of nicotinamide, which is a well known precursor of nicotinamide-adenine dinucleotide (NAD⁺) in animals. On the other hand, the metabolic reduction of other N-oxide compounds has also been reported. In the enzymes capable of catalyzing the N-oxide reduction was isolated from hog liver and identified as xanthine oxidase by Murray and Chaykin. Recently, Kitamura and Tatsumi found that aldehyde oxidase (EC 1.2.3.1) rather than xanthine oxidase is the major enzyme responsible for the reduction of nicotinamide N-oxide in mammalian livers. However, no reports are available which describe the metabolic reduction of nicotinamide N-oxide in extrahepatic tissues. The aldehyde oxidase-dependent reaction is as follows: 2-hydroxypyrimidine + nicotinamide N-oxide → uracil + nicotinamide.

We report here the first description of tissue localization of nicotinamide N-oxide reductase activity in the eye. We demonstrate the participation of aldehyde oxidase in the N-oxide reduction catalyzed by ciliary body, retinal pigment epithelium-choroid, iris, retina and cornea preparations.

Materials and Methods. Tissue preparation. Fresh bovine eyes were obtained from a local slaughterhouse and brought to the laboratory on ice. Rabbit eyes were removed from albino rabbits in the laboratory just prior to use. Cornea, lens, iris, ciliary body, retina and retinal pigment epithelium-choroid were dissected out separately. Subcellular fractionation of ocular tissues was performed with a modification of the method reported by Das and Shichi, as follows: the tissue was homogenized with four volumes of 0.02 M Tris-HCl buffer (pH 7.4) containing 0.25 M sucrose, first in a Polytron and then in a Potter-Elvehjem homogenizer. The homogenate was then centrifuged for 20 min at 9,000g. In the case of ciliary body, the 9,000g supernatant was further centrifuged for 60 min at 105,000g to separate the cytosol from microsomes. The microsomes were washed by resuspension in the Tris-HCl buffer and resedimentation.
Nicotinamide N-oxide reductase activity of 9,000g supernatants from a variety of bovine ocular tissues

Table 1. Nicotinamide N-oxide reductase activity of 9,000g supernatants from a variety of bovine ocular tissues

<table>
<thead>
<tr>
<th>Addition</th>
<th>Lens</th>
<th>Cornea</th>
<th>Retina</th>
<th>RPE*-choroid</th>
<th>Iris</th>
<th>Ciliary body</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2-hydroxyprymidine</td>
<td>0</td>
<td>110</td>
<td>290</td>
<td>4,450</td>
<td>3,610</td>
<td>11,710</td>
</tr>
<tr>
<td>NADPH</td>
<td>50</td>
<td>0</td>
<td>80</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>NADH</td>
<td>0</td>
<td>30</td>
<td>30</td>
<td>70</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Xanthine</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>40</td>
</tr>
</tbody>
</table>

Each value represents the mean of four experiments.

*Retinal pigment epithelium.

The incubation mixture consisted of 0.5 μmol of nicotinamide N-oxide, 2 μmol of an electron donor and the sample tissue preparation in a final volume of 1 ml of 0.1 M Tris-HCl buffer (pH 7.4). The mixture was incubated using a Thunberg tube for 10-min at 37°C under an atmosphere of nitrogen; the reaction was stopped by adding 0.1 ml of 5N NaOH. A control tube lacking nicotinamide N-oxide was run with each reaction tube to correct for formation of nicotinamide other than that from nicotinamide N-oxide. After adding 30 μg of benzamide as an internal standard and 0.1 g of NaCl, the mixture was extracted twice with 5 ml each of ethyl acetate and the combined extract was evaporated to dryness in vacuo. The residue was dissolved in 0.1 ml of methanol and then subjected to high-pressure liquid chromatography (HPLC). HPLC was performed in a Toyo Soda HLC-803A chromatograph equipped with a UV-8 UV absorption detector. The instrument was fitted with a 15-cm × 4.6-mm (I.D.) M & S Pack C18 column. The mobile phase was methanol-water (15:85). The chromatogram was operated at a flow rate of 0.80 ml/min at ambient temperature and at a wavelength of 254 nm. The nicotinamide (elution time 5.7 min) formed was determined from its peak area.

Results. The ability to reduce nicotinamide N-oxide to nicotinamide was examined with a variety of bovine ocular tissues. As shown in Table 1, the 9,000g supernatant of ciliary body, retinal pigment epithelium-choroid, iris, retina and cornea, but not lens, exhibited nicotinamide N-oxide reductase activity under anaerobic conditions in varying degrees when supplemented with 2-hydroxyprymidine, an electron donor of aldehyde oxidase. Among these tissues, the highest activity was observed with ciliary body, followed by retinal pigment epithelium-choroid and iris. However, only slight or no reductase activity was observed with NADPH, NADH or xanthine, which is an electron donor for the cytochrome P-450 system, DT-diaphorase or xanthine oxidase. On the other hand, the 9,000g sediment of each tissue showed no ability to reduce nicotinamide N-oxide even in the presence of the electron donors described above.

When the 9,000g supernatant of ciliary body was further fractionated by centrifugation, the 2-hydroxyprymidine-linked reductase activity was mainly associated with the cytosolic fraction and markedly inhibited by menadione (1 × 10⁻⁴ M), chlorpromazine (2 × 10⁻⁴ M) or amidol (2 × 10⁻⁴ M), which are inhibitors of aldehyde oxidase. The cytosolic fraction also exhibited nicotinamide N-oxide reductase activity, but to a lesser extent, when supplemented with other electron donors of aldehyde oxidase, such as N¹-methylnicotinamide and benzaldehyde. However, little or no reductase activity was observed with the microsomal fraction in the presence of NADPH or NADH.

The ability of rabbit ciliary body to reduce nicotinamide N-oxide was also examined in the same manner. Its cytosol with 2-hydroxyprymidine exhibited reductase activity comparable to that shown by bovine ciliary body as described above. The activity was again markedly inhibited by menadione (1 × 10⁻⁴ M).

These results strongly suggest that aldehyde oxidase present in mammalian eyes is involved in the reduction of nicotinamide N-oxide.

Discussion. In the present study, no attempt was made to purify aldehyde oxidase from ocular tissues and directly examine its ability to reduce nicotinamide N-oxide. However, a recent study done by Kita and Tatsumi showed that rabbit liver aldehyde oxidase, in the presence of an electron donor such as 2-hydroxyprymidine and under anaerobic conditions, exhibits a significant nicotinamide N-oxide reductase activity which is markedly inhibited by menadione. The enzyme is located in the liver cytosolic fraction, which also exhibits N-oxide reductase activity when supplemented with an electron donor specific for the enzyme. These facts support the assumption that nicotinamide N-oxide reductase activity detected in ocular tissues, especially in the ciliary body, is mainly due to aldehyde oxidase.

Previously, Das and Shichi reported the highly specific localization of some drug-metabolizing enzyme activities in bovine eyes: the highest activities of aryl hydrocarbon hydroxylase and UDPglucuronosyltransferase were found in the ciliary body, followed by the retinal pigment epithelium-choroid and iris. Mercapturic acid-synthesizing enzyme activities were also associated with these tissues. The present study, however, showed a similar localization of nic-
otinamide N-oxide reductase activity in bovine eyes, suggesting that the ocular tissues described above are rich in enzyme activities responsible for not only drug metabolism but also intermediary metabolism.

As regards aldehyde oxidase, only the liver enzyme has been extensively characterized. Its molecular weight has been estimated to be about 300,000. This liver enzyme, which contains molybdenum, iron and FAD in its molecule, can catalyze the oxidation of both aldehydeic and N-heterocyclic compounds, and can utilize electron acceptors such as molecular oxygen and potassium ferricyanide. In the field of drug metabolism, this liver enzyme has been recognized as the major reductase responsible for the reduction of a variety of xenobiotics, such as sulfoxides, nitrosoamines, azo dyes, N-oxides, aromatic nitro compounds, and hydroxamic acids.

**Key words:** bovine, rabbit, eye, metabolic reduction, nicotinamide N-oxide, aldehyde oxidase

From the Department of Ophthalmology and the Institute of Pharmaceutical Sciences, Hiroshima University School of Medicine, Hiroshima, Japan. Submitted for publication: September 18, 1986. Reprint requests: Shigeaki Shimada, Department of Ophthalmology of Hiroshima University School of Medicine, 1-2-3, Kasumi, Minami-ku, Hiroshima 734, Japan.

**References**


**Methotrexate-Anticollagen Conjugate Inhibits In Vitro Lens Cell Outgrowth**

Thomsen J. Hansen,*† Roxane Tyndall,† and David D. Sol‡

After-cataract, or posterior lens capsule opacification, is an undesirable but common sequela to extracapsular cataract surgery. We are investigating biochemical means to prevent after-cataract formation, which can be applied at the time of the original surgery. Based on similar research efforts in cancer chemotherapy, we have prepared a conjugate of the antimetabolic agent methotrexate with an antibody specific for basement membrane collagen, the major protein in the lens capsule. The conjugate was evaluated using biochemical measurements, and retained both antimetabolic and antibody activities. When the conjugate was applied to bovine posterior capsules in vitro, or in vivo in rabbits, it was an effective inhibitor of lens epithelial cell outgrowth in cell culture. Invest Ophthalmol Vis Sci 28:1206-1209, 1987

Extracapsular lens extraction has become the method of choice for removing cataracts. The major medical advantages of this technique over intracapsular extraction are lower incidences of both aphakic cystoid macular edema and retinal detachment. Extracapsular extraction is also required for implantation of posterior chamber-type intraocular lenses, which are now considered to be the lenses of choice in most cases. A disadvantage of extracapsular cataract extraction is the high incidence of posterior lens capsule opacification, often called after-cataract, which can occur in up to 50% of cases within 3 yr after surgery. After-cataract is caused by proliferation of equatorial and anterior capsule lens epithelial cells which remain after extracapsular lens extraction. This can be treated by additional surgery, but prevention of after-cataract would be preferable to treatment, and could be achieved by physically or chemically destroying the lens cells at the time of the original cataract extraction.

In animal experiments, cytotoxic drugs administered during surgery or intraperitoneally have been investigated as a means of inhibiting lens epithelial cells. Of these, the most successful has been methotrexate (MTX). MTX kills dividing cells preferentially, though not exclusively, and is used in cancer chemotherapy. Because MTX is not specific as to the type of cell that it kills, serious side effects can occur. Targeting MTX to cancer cells has been attempted by covalently linking the drug to an antibody specific for that cell type. Targeting simultaneously allows a greater concentration of MTX in the