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Aldose Reductase, a Key Enzyme in the Oxidative Deamination of Norepinephrine in Rats

Minoru Kawamura,† Graeme Eisenhofer,* Irwin J. Kopin,* Peter F. Kador,‡ Yong S. Lee,‡ Jen-Yue Tsai,‡ Shigeki Fujisawa,‡ Martin J. Lizak,‡ Andrea Sinz‡ and Sanai Sato‡§*

*Clinical Neuroscience Branch, National Institute of Neurological Disorders and Stroke and ‡Laboratory of Ocular Therapeutics, National Eye Institute, National Institutes of Health, Bethesda, MD 20892, U.S.A.; and †Institute of Bio-Active Science, Nippon Zoki Pharmaceutical Co., Ltd., Hyogo 673–14, Japan

ABSTRACT. The sympathoneural neurotransmitter norepinephrine (NE) is deaminated to 3,4-dihydroxymandelaldehyde (DHMAL) and subsequently converted to either 3,4-dihydroxymandelic acid (DHMA) or 3,4-dihydroxyphenylglycol (DHPG). In this study, we investigated the relative importance of aldose reductase versus aldehyde reductase in the formation of DHPG from DHMAL. The *in vitro* incubation of NE with aldose reductase in the presence of monoamine oxidase (MAO) resulted in the formation of DHPG, which was confirmed by mass spectrometry. Although aldehyde reductase also generated DHPG, its activity was much lower than that of aldose reductase. With northern blotting, the expression of both aldose reductase and aldehyde reductase was detected in rat superior cervical ganglia. However, with western blotting, only aldose reductase was immunologically detectable. Treatment of rats with aldose reductase inhibitors for 3 days increased the plasma level of DHMA. There was no correlation between the selectivity of inhibitors and effects on NE metabolite levels. A significant decrease in DHPG, however, was obtained only with an extremely high dose (9 mg/kg/day) of the nonselective inhibitor AL 1576. The present study confirmed that aldose reductase generates DHPG from NE in the presence of MAO. In rat sympathetic neurons, aldose reductase appears to be more important than aldehyde reductase for the formation of DHPG. However, when aldose reductase is inhibited, it appears that aldehyde reductase can compensate for the conversion of DHMAL to DHPG, indicating redundancy in the reduction pathway. BIOCHEM PHARMACOL **58**;3:517–524, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. aldose reductase; aldehyde reductase; norepinephrine; 3,4-dihydroxymandelaldehyde; 3,4-dihydroxyphenylglycol; sympathetic nervous system

There are two major pathways for catecholamine metabolism, oxidative deamination by MAO‖ (EC 1.4.3.4) and *O*-methylation by catechol-*O*-methyltransferase (EC 2.1.1.6). Because MAO is the only enzyme present at intraneural locations [1], the major flux of NE metabolism in sympathetic neurons is the formation of DHMAL by MAO. This aldehyde intermediate is metabolized further into either the oxidation product, DHMA, or the reduction product, DHPG. It generally is accepted that biogenic amines such as NE that contain a hydroxyl group adjacent to the amine function are metabolized mainly to glycol products, whereas the main products from those lacking the hydroxyl group, e.g. dopamine, are the acid metabolites [2]. As a result, the intraneural metabolism of NE involves almost exclusive formation of DHPG through the aldehyde intermediate by MAO (see the review by Kopin [3] for details).

Two enzymes, aldose reductase (alditol:$NADP^+$ 1-oxidoreductase, EC 1.1.1.21) and/or aldehyde reductase (alcohol:$NADP^+$ oxidoreductase, EC 1.1.1.2), are responsible for the reduction of the aldehyde intermediate from NE to DHPG [4–7]. The kinetic properties with aldehydes from biogenic amines appear to be more favorable to aldose reductase than to aldehyde reductase [4, 7–9]. Whittle and Turner [10] also reported that the aldehyde reductase inhibitor sodium valproate does not affect the formation of 3-methoxy-4-hydroxyphenylglycol in rat brain, suggesting that aldose reductase plays a major role in biogenic aldehyde metabolism. However, despite the above evidence, aldehyde reductase still is commonly considered and cited as the enzyme responsible for the formation of DHPG. Even in a recent review article [11], aldehyde reductase is described as the enzyme that plays a significant role in the metabolism of neurotransmitter aldehydes. Moreover, the presence of aldose reductase and/or aldehyde reductase in sympathetic neurons, the major source of NE, has not been

§ Corresponding author: Sanai Sato, M.D., LOT/NEI/NIH, 10/10B09, 10 Center Dr., MSC 1850, Bethesda, MD 20892-1850. Tel. (301) 402-9849; FAX (301) 402-2399; E-mail: sanai@helix.nih.gov

‖ *Abbreviations*: DHBA, 3,4-dihydroxybenzylamine; DHMA, 3,4-dihydroxymandelic acid; DHMAL, 3,4-dihydroxymandelaldehyde or 3,4-dihydroxyphenylglycolaldehyde; DHPG, 3,4-dihydroxyphenylglycol; ES-MS, electrospray mass spectrometry; MAO, monoamine oxidase; NE, norepinephrine; and SCG, superior cervical ganglia.

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confirmed. In this study, the metabolite from NE generated by aldose reductase was identified as DHPG using an *in vitro* incubation system. The localization of aldose reductase and aldehyde reductase in rat SCG and the relative importance of aldose reductase versus aldehyde reductase in DHPG formation were examined with aldose reductase inhibitors with different selectivity.

MATERIALS AND METHODS

Chemicals

NE bitartrate, DHPG, DHMA, DHBA, and pargyline were all obtained from the Sigma Chemical Co. Matrex Gel Orange A was a product of the Amicon Corp. Prestained molecular weight protein standards for SDS–PAGE and peroxidase substrate (4-chloro-1-naphthol and hydrogen peroxide) were purchased from Bethesda Research Laboratories Life Technologies Inc. and Kirkegaard & Perry Laboratories, Inc., respectively. 3-Deoxy-3-fluoro-D-glucose was obtained from Omicron Biochemicals, Inc. The aldose reductase inhibitors AL 1576 (2,7-difluorospirofluorene-9,5′-imidazolidine-2′,4′-dione; Imirestat), tolrestat (*N*-[[5-(trifluoromethyl)-6-methoxy-1-naphthalenyl]thioxomethyl]-*N*-methylglycine), and Ponalrestat [3-(4-bromo-2-fluorobenzyl)-4-oxo-3-phthalazine-1-yl acetic acid] were gifts from Alcon Laboratories, Wyeth-Ayerst, and ICI Americas, respectively.

Animals

Male Sprague–Dawley rats were purchased from Taconic Farm. Animals weighing 244–347 g were utilized for *in vivo* experiments to assess the effects of aldose reductase inhibitors on plasma DHPG and DHMA levels and 3-fluoro-3-deoxy-D-sorbitol formation in red blood cells. Rats were divided into seven groups, consisting of 4–9 rats each, and fed with diets containing 0.0016 and 0.016% of AL 1576 or 0.05 and 0.5% of tolrestat or Ponalrestat for 3 days. The doses of drugs administered to rats were expressed as milligrams per kilogram per day, using the drug concentrations in their diet and mean values of their food intake and body weight during 3 days of administration. One of the groups received a normal diet as the control group with the same schedule as those of the aldose reductase inhibitor-treated groups. After 3 days of scheduled feeding, 50 mg of 3-fluoro-3-deoxy-D-glucose dissolved in 1 mL of distilled water was injected into rats through a tail vein twice at 3 and 1.5 hr before blood sampling. Blood samples (4 mL) were collected from the abdominal aorta into heparinized tubes under anesthesia by i.p. injection of sodium pentobarbital (50–65 mg/kg). The collected blood samples were frozen immediately on dry ice and stored at −80° for NMR analysis. For DHPG and DHMA assays, blood was centrifuged to separate plasma, and the plasma was stored at −80°.

All tissues utilized for northern and western blot analyses were obtained from normal control rats weighing 247–379 g. Rats were dissected under pentobarbital anesthesia (60 mg/kg, i.p. injection), and tissues were frozen immediately on dry ice and stored at −80° until processed.

Enzyme Assay

Reductase activity was assayed spectrophotometrically by following the decrease of NADPH at 340 nm for 4 min. The reaction mixture consisted of 1 mL of phosphate buffer, pH 6.2, containing 2–3 mU of enzyme, 0.3 mM NADPH, and 10 mM DL-glyceraldehyde. One enzyme unit (U) was defined as the activity consuming 1 μmol of NADPH/min at 22°.

Preparation of Rat Lens Aldose Reductase and Rat Liver Aldehyde Reductase

Aldehyde reductase was purified from rat liver by slightly modifying the previously reported chromatographic procedure [12]. Briefly, 1 g of rat liver was homogenized with 5 mL of 20 mM imidazole-HCl buffer, pH 7.5, containing 10 mM 2-mercaptoethanol. After centrifugation at 10,000 *g* for 10 min, the supernatant was fractionated with 30–60% ammonium sulfate. The precipitate was dissolved in a minimum volume of imidazole buffer, centrifuged at 10,000 *g* for 10 min, and the supernatant then was applied to a Sephacryl S-100 column (HiPrepTM 16/60, Pharmacia-LKB Biotechnology, Inc.). The column was developed with imidazole buffer at a flow rate of 2 mL/min, and the eluent was collected into 100-drop aliquots (approx. 5 mL). Fractions containing reductase activity were collected and applied to a Matrex Gel Orange A column (1.25 × 20 mm). The column was washed with 300 mL of imidazole buffer, and the enzyme was eluted with the same imidazole buffer containing 0.1 mM NADPH. Fractions eluted with NADPH were chromatofocused on a Mono P column (HR 5/20, Pharmacia-LKB Biotechnology, Inc.) equilibrated with 20 mM imidazole-HCl buffer, pH 7.5, containing 10 mM 2-mercaptoethanol. The column was developed at a flow rate of 1 mL/min with 50 mL of Polybuffer 74, which was diluted 10-fold with 10 mM 2-mercaptoethanol. The protein concentration of eluent was monitored at 280 nm, and the peak of aldehyde reductase was collected and concentrated with a Centricon 10 system (Amicon, Inc.) The specific activity of the purified aldehyde reductase with DL-glyceraldehyde as substrate was 4.2 U/min/mg protein.

The source of aldose reductase utilized in this study was recombinant rat lens aldose reductase expressed in *Escherichia coli*, which was isolated as previously reported [13]. The specific activity was 2.5 U/min/mg protein with DL-glyceraldehyde as substrate.

SDS–PAGE and Immunoblotting

SDS–PAGE was performed on 10% acrylamide gel according to the method of Laemmli [14] using the Novex El9001-XCELL II Mini Cell system (Novex). Phosphory-

lase *b* (94 kDa), BSA (67 kDa), ovalbumin (43 kDa), and carbonic anhydrase (30 kDa) were used as standards. Proteins were stained with Coomassie blue.

For the immunoblotting, proteins were transferred from acrylamide gel onto a nitrocellulose membrane (Bio-Rad Laboratories) using a Panther Semi-Dry Electroblotter (Owl Scientific). Nonspecific binding onto the nitrocellulose membrane was blocked with 5% nonfat dry milk solution dissolved with PBS for 2 hr at room temperature. The nitrocellulose membrane then was incubated overnight at 4° in PBS containing antisera (200-fold dilution) raised in goat against either purified rat lens aldose reductase [15] or rat kidney aldehyde reductase [12]. After three washings with PBS, the membrane was incubated with the second antibody (biotinylated rabbit IgG against goat IgG) and then with a mixture of avidin and biotinylated peroxidase using a Vectastain ABC kit (Vector Laboratories, Inc.). The immunostaining was visualized by peroxidase reaction with 4-chloro-1-naphthol.

RNA Analysis

Total RNAs were extracted from frozen tissues by the guanidinium chloride/phenol method using the Trizol reagent (GIBCO/BRL Life Technology). Ten micrograms of total RNA from each tissue then was subjected to northern blot analysis according to standard procedures [16]. Ethidium bromide (final concentration 0.01 μg/μL) was added to the RNA samples before loading to visualize the ribosomal RNA bands. Purified rat lens aldose reductase cDNA and mouse liver aldehyde reductase cDNA fragments were labeled with [^{32}P]dCTP using a random primer labeling kit (Pharmacia-LKB Biotechnology, Inc.). Hybridization was performed at 65° in Church buffer [17] for 12 hr, and the blot was washed twice at 65° with 0.1x SSC (0.15 M sodium chloride + 0.015 M sodium citrate, pH 7.0), 0.1% SDS for 30 min each.

Preparation of Mitochondrial Monoamine Oxidase

Mitochondria were isolated from rat liver homogenate by a series of centrifugations as described previously [18]. Rats were anesthetized by i.p. injection of pentobarbital (60 mg/kg) and perfused via the heart with ice-cold isolation medium (220 mM d-mannitol, 70 mM sucrose, 5 mM MOPS, and 2 mM EDTA, adjusted to pH 7.4 by KOH). Livers were homogenized immediately with ice-cold isolation medium using a Potter–Elvehjem homogenizer. After centrifugation at 900 *g* for 4 min at 4°, the supernatant was centrifuged at 10,000 *g* for 8 min. The sediment was washed once by resuspending with ice-cold isolation medium and centrifuging again at 10,000 *g* for 8 min. The sediment (mitochondrial fraction) was resuspended in ice-cold 50 mM potassium phosphate buffer (pH 7.3) and sonicated with a Misonix Ultrasonic Processor XL. After centrifugation at 100,000 *g* for 60 min, the pellet (mitochondrial membrane fraction) was resuspended with 50 mM potassium phosphate buffer (pH 7.3) and stored at −80°. This suspension of rat liver mitochondrial membrane fraction was utilized as MAO in this study.

In Vitro *Incubation*

Unless otherwise stated, the reaction mixture (100 μL) consisted of 0.1 M phosphate buffer, pH 7.4, 2 mM ascorbic acid, 100 μM NE, 500 μM NADPH, 10 μL MAO, and aldose reductase (25 mU assayed using DL-glyceraldehyde as substrate). The reaction was started by adding MAO. The mixture was incubated at 37° for 3 hr and terminated by the addition of 50 μL of 0.4 M perchloric acid containing 0.5 mM EDTA. After centrifugation at 10,000 *g* for 15 min at 4°, the supernatant was stored at −80° for the catechol assay.

For the analysis by ES-MS, the reaction was stopped by filtering with a Microcon SR3 (molecular weight 3000 cut filter; Amicon, Inc.) after the incubation at 37° for 3 hr.

HPLC

Identification and quantification of catechols were performed by HPLC with electrochemical detection as previously described [19]. One hundred microliters of sample, appropriately diluted with 0.2 M acetic acid, was mixed with 20 μL (14.37 pmol) of DHBA (a catechol internal standard), 10 mg of alumina, and 800 μL of 1 M Tris–HCl buffer, pH 8.6, containing 50 mM EDTA. Catechols were extracted with 100 μL of mobile phase (0.1 M NaH_2PO_4, 0.13 mM EDTA, 0.23 mM octane sulfonate, 0.4% acetonitrile, pH 3.13), adjusted to pH 2.2 with phosphoric acid, and analyzed by HPLC using a 5-μm particle size Axxichrom C18 reversed-phase column (250 × 4.6 mm i.d.) (Axxiom Chromatography). Catechols were identified according to appropriate authentic standards.

For ES-MS analysis, H_2O with 1% trifluoroacetic acid was utilized as the mobile phase. The metabolite peaks were detected at 280 nm.

Aldose Reductase Assay in Vivo *with 3-Fluoro-3-deoxy-D-glucose*

Aldose reductase activity in red blood cells was estimated by the formation of 3-fluoro-3-deoxy-D-sorbitol in whole blood. Blood 3-fluoro-3-deoxy-D-sorbitol was measured by ^{19}F-NMR spectroscopy using a General Electric Omega NMR spectrophotometer as described by Berkowitz *et al.* [20] and Karino *et al.* [21].

RESULTS

DHPG Formation by Aldose Reductase

The formation of DHPG from NE was investigated with an *in vitro* incubation system containing NE, MAO, and aldose reductase (Fig. 1). Incubation without MAO and aldose reductase resulted in peaks corresponding to unmetabolized

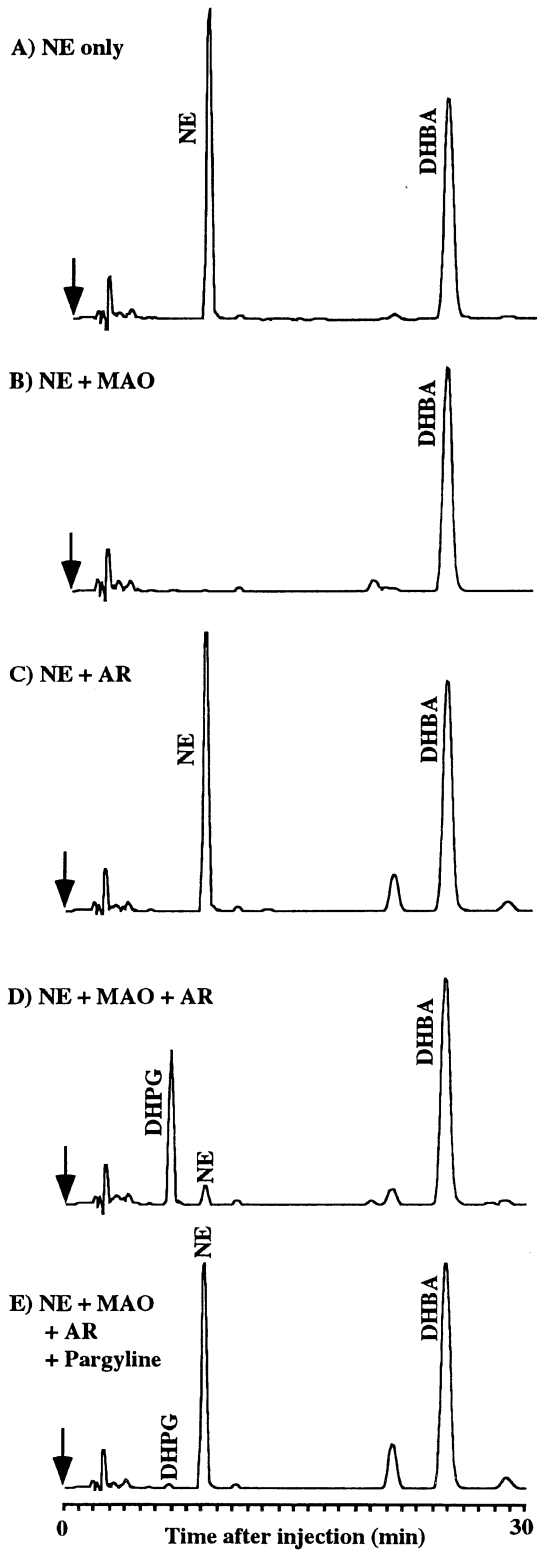

FIG. 1. DHPG formation by aldose reductase. The reaction mixture described in Materials and Methods was incubated at 37° for 3 hr. Panels A, B, C, D, and E represent the chromatograms obtained from the reaction mixture of NE without both aldose reductase (AR) and MAO, the mixture without AR, the mixture without MAO, the complete reaction mixture containing both AR and MAO, and the complete reaction mixture containing a 1 mM concentration of the MAO inhibitor pargyline, respectively.

NE and DHBA, an internal standard added to the reaction mixture after the incubation. When MAO was added to the mixture, the NE peak disappeared, indicating that MAO almost completely metabolized the amine. However, DHPG was not detected. The mixture containing NE and aldose reductase displayed only the NE peak. This profile was virtually identical to that of control NE. The reaction mixture containing both MAO and aldose reductase displayed the clear peak of the newly formed DHPG, confirming that DHPG formation occurs only in the presence of both MAO and aldose reductase. The addition of an MAO inhibitor, pargyline, into the complete mixture significantly reduced the peak of DHPG. Pargyline also prevented the decrease of the NE peak, confirming that pargyline reduced DHPG formation by inhibiting the metabolism of NE by MAO. Based on the peak height, the inhibition of MAO by 1 mM pargyline was approximately 80–90%.

The formation of DHPG through the intermediate aldehyde, DHMAL, also was confirmed by detecting both DHPG and DHMAL in the reaction mixture by ES-MS (Fig. 2).

The effects of aldose reductase inhibitors on DHPG formation also were investigated with three structurally diverse inhibitors: AL 1576, tolrestat, and Ponalrestat (Table 1). Among these three compounds, AL 1576 was the most potent, displaying 93 and 96% inhibition at 1 and 10 μM, respectively. Ponalrestat was the least potent among the three inhibitors, with an inhibition of 87% at 1 μM.

DHPG Formation by Aldehyde Reductase

The reaction mixture containing aldehyde reductase instead of aldose reductase also displayed formation of DHPG. To compare aldose reductase and aldehyde reductase, we examined the formation of DHPG by the same amount of enzyme activity assayed with DL-glyceraldehyde as substrate (Fig. 3). The DHPG concentrations produced by 0.001 and 0.01 mU of aldose reductase were 6.5 and 35.9 pmol/mL, whereas those of 0.01, 0.1, and 1 mU of aldehyde reductase were 5.6, 14.8, and 109.6 pmol/mL, respectively. This indicated that aldose reductase was more active than aldehyde reductase in producing DHPG. The specific activity (4.2 U/min/mg protein) of aldehyde reductase utilized in this experiment was approximately 2-fold higher than that (2.5 U/min/mg) of aldose reductase. This indicates that, even when comparisons were based on the amount of enzyme as protein, aldose reductase was almost 5-fold more active than aldehyde reductase.

Localization of Aldose Reductase and Aldehyde Reductase in Rat SCG

To establish the presence of aldose reductase in sympathetic nerve cells, both northern and western blots were conducted with rat SCG. With the northern blot (Fig. 4), all tissues examined displayed strong staining for aldose

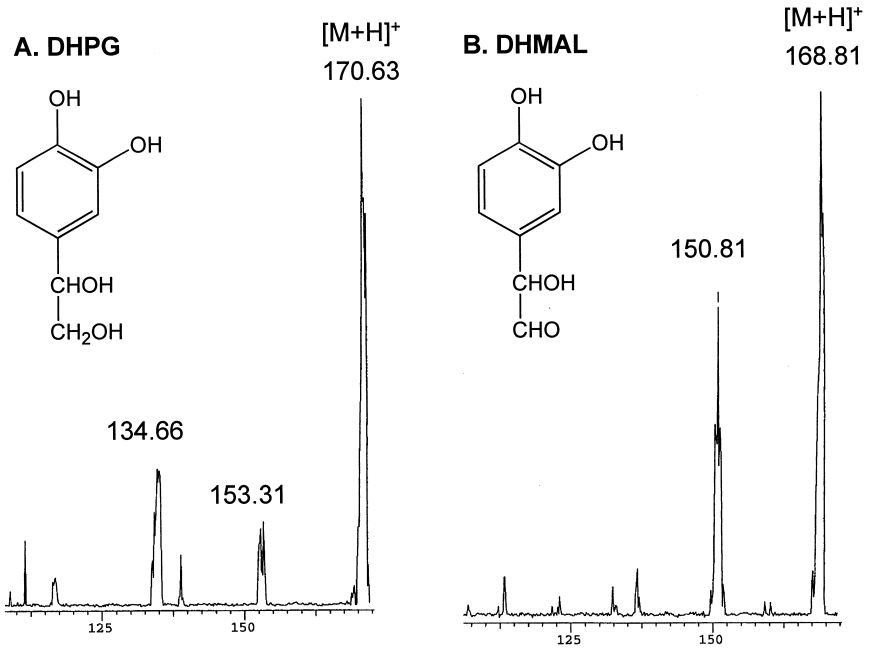

FIG. 2. ES-MS analysis of the metabolites generated in the complete incubation mixture containing NE, aldose reductase, and MAO. Spectrum A indicates DHPG with the mother peak at *m/z* 170. Loss of one hydroxyl group yields an ion at *m/z* 153. Another loss of a hydroxyl group yields a fragment ion at 134. Spectrum B indicates DHMAL. The mother peak is at *m/z* 168 with a fragment ion at 150 generated by the loss of one hydroxyl group.

reductase except for the kidney. The SCG also displayed high expression levels for aldose reductase. Compared with other tissues, this tissue also displayed relatively strong staining for aldehyde reductase. This confirmed that both aldose reductase and aldehyde reductase were expressed in rat SCG. However, the levels of translation products appeared to be different. In western blots (Fig. 5), crude SCG extracts gave a positive band with antibody against rat lens aldose reductase, which exactly corresponded to purified aldose reductase. In contrast, the same crude extract of rat SCG gave no recognizable positive band with antibody against rat kidney aldehyde reductase despite positive staining with purified rat liver aldehyde reductase.

Effects of Aldose Reductase Inhibitors on Sorbitol Formation and NE Metabolism in Vivo

Rats fed a diet containing the aldose reductase inhibitors AL 1576, tolrestat, and Ponalrestat for 3 days displayed significant decreases of 3-fluoro-3-deoxy-D-sorbitol formation in whole blood and increased plasma DHMA levels (Table 2). Among these three inhibitors, AL 1576 displayed the strongest inhibition of sorbitol formation in whole blood. With the low dose (1 mg/kg/day) of AL 1576, the formation of 3-fluoro-3-deoxy-D-sorbitol was almost undetectable. Based on the effect on the polyol formation, the low dose of AL 1576 was considered to be equivalent in inhibiting aldose reductase to the high doses of tolrestat and Ponalrestat (301 and 344 mg/kg/day, respectively).

As previously reported [22], the increase of DHMA is a more sensitive marker of the effects of aldose reductase inhibitors on NE metabolism than is the decrease of DHPG. All three inhibitors increased the plasma levels of DHMA in a dose-dependent manner. These three inhibitors differ in their selectivity for inhibition of aldose reductase versus aldehyde reductase [23]. Whereas AL 1576 almost equally inhibited both aldose reductase and alde-

TABLE 1. Effects of aldose reductase inhibitors on DHPG formation aldose reductase

Inhibitor	Concentration (μM)	DHPG (pmol/mL)	Inhibition (%)
No inhibitor		20.1 ± 2.6*	
AL 1576	1	1.46 ± 0.08	92.7
	10	0.85 ± 0.09	95.8
Tolrestat	1	1.33 ± 0.04	93.4
	10	1.39 ± 0.10	93.1
Ponalrestat	1	2.63 ± 0.12	86.9
	10	2.19 ± 0.05	89.1

*Mean ± SD (N = 4).

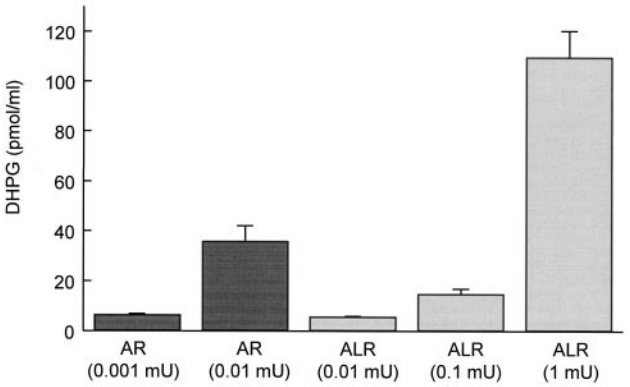

FIG. 3. DHPG formation by 0.001 and 0.01 mU of aldose reductase (AR) and 0.01, 0.1, and 1 mU of aldehyde reductase (ALR). The enzyme unit (U) is expressed as micromoles per minute per milliliter with DL-glyceraldehyde as substrate. Data are means ± SD (N = 4).

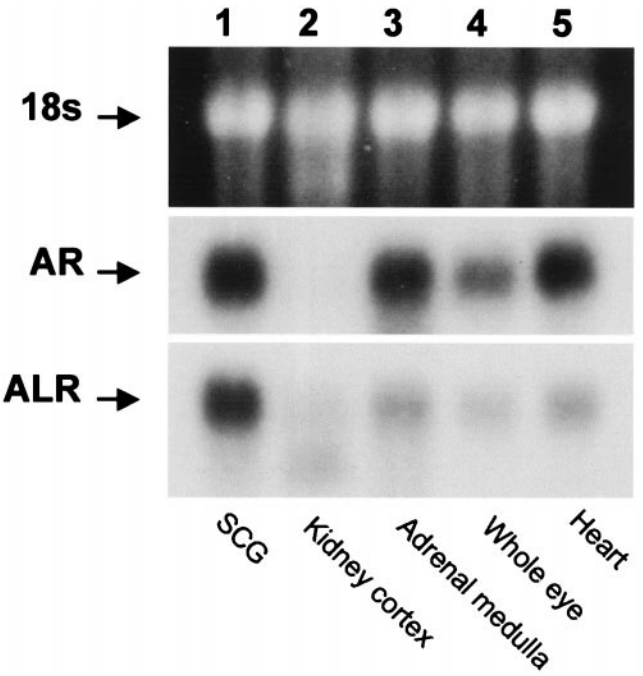

FIG. 4. Northern blots of rat SCG (lane 1), kidney cortex (lane 2), medulla of adrenal gland (lane 3), whole eye (lane 4), and heart (lane 5) with rat lens aldose reductase cDNA (AR) and mouse liver aldehyde reductase cDNA (ALR).

hyde reductase, tolrestat and Ponalrestat were 22- and 119-fold more selective for aldose reductase than aldehyde reductase. Despite the large differences in selectivity, the increase in plasma DHMA by the low dose of AL 1576 was almost equivalent or even less than the increase caused by the two selective inhibitors, tolrestat and Ponalrestat, at doses producing equivalent inhibition of aldose reductase (as reflected by $> 95\%$ inhibition of sorbitol formation). This clearly indicates that there is no correlation between inhibitor selectivity and an effect on the increase in plasma DHMA levels.

When the dose of AL 1576 was increased to 9 mg/kg/day, a significant decrease of DHPG was observed.

DISCUSSION

The major first step of intraneural metabolism of NE is oxidative deamination by MAO. This step terminates the physiological role of NE as a neurotransmitter and generates the potentially cytotoxic aldehyde intermediate DHMAL [24]. Since the major metabolite formed by this step is the reduction product, DHPG, rather than the oxidation product, DHMA, either aldose reductase or aldehyde reductase must play a major role in the removal of the potentially cytotoxic aldehyde intermediate. The present data indicate that, although both aldose reductase and aldehyde reductase may contribute to the reduction of DHMAL to DHPG, aldose reductase appears to be the more important enzyme in NE metabolism in rats.

When NE was incubated *in vitro* with aldose reductase in the presence of MAO and NADPH, DHPG was formed. The metabolite from NE by aldose reductase was confirmed to be DHPG, not only by the elution profile from HPLC, but also by MS. DHMAL also was generated in the incubation mixture. This clearly indicates that aldose reductase generates DHPG by reducing DHMAL formed from NE by MAO. As far as assessed by the formation of DHPG under these *in vitro* conditions, aldose reductase is more active than aldehyde reductase. This is consistent with the published evidence that kinetic properties with various biogenic aldehyde substrates are more favorable to aldose reductase than to aldehyde reductase [4, 7–9]. The present study also confirmed that aldose reductase is present in sympathetic neurons. Although aldehyde reductase is also present in this tissue, aldose reductase appears to be the dominant enzyme. When combined, the data of different activity and localization in sympathetic neurons indicate that aldose reductase, rather than aldehyde reductase, is the primary enzyme responsible for the formation of DHPG in rats.

A number of aldose reductase inhibitors with diverse structures have been developed. Many of these, to some extent, also inhibit aldehyde reductase [23, 25]. Taking advantage of differences in selectivity, the importance of aldose reductase versus aldehyde reductase in the *in vivo* metabolism of NE also was assessed with three aldose reductase inhibitors. If aldehyde reductase is important in NE metabolism, then the nonselective inhibitor AL 1576, at concentrations inhibiting sorbitol formation to the same

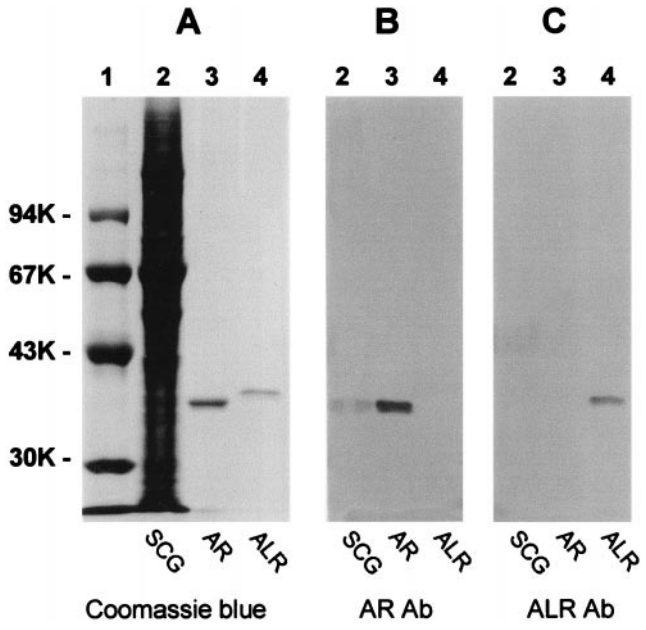

FIG. 5. Western blot of the crude extract of rat SCG (50 μg of protein) (lane 2), rat lens aldose reductase (AR, 0.2 μg of protein) (lane 3), and rat liver aldehyde reductase (ALR, 0.2 μg of protein) (lane 4). Panel A represents protein staining with Coomassie blue. Panels B and C represent immunoblots with antibodies against rat lens aldose reductase and rat kidney aldehyde reductase, respectively. For the immunoblots, 0.05 μg of proteins of purified enzymes was applied. Lane 1: molecular weight standards.

TABLE 2. Effects of aldose reductase inhibitors on 3-fluoro-3-deoxy-D-sorbitol formation in red blood cells and on plasma NE metabolites

Groups	Dose (mg/kg/day)	Sorbitol in red blood cells (% inhibition)	Plasma DHMA (pmol/mL)	Plasma DHPG (pmol/mL)
Control			0.07 ± 0.02 (N = 8)	4.85 ± 0.63 (N = 9)
AL 1576 (1:2)*	1	>95† (N = 6)	0.77 ± 0.45‡ (N = 6)	4.86 ± 0.95 (N = 6)
	9	>95† (N = 5)	4.43 ± 0.78‡§ (N = 7)	1.88 ± 0.37‡§ (N = 7)
Tolrestat (1:22)*	36	76 ± 9 (N = 4)	0.77 ± 0.53† (N = 6)	4.49 ± 1.54 (N = 6)
	301	>95† (N = 6)	1.94 ± 0.74‡ (N = 6)	4.28 ± 0.55 (N = 6)
Proalrestat (1:119)*	36	84 ± 18 (N = 6)	0.29 ± 0.17 (N = 6)	5.06 ± 0.46 (N = 6)
	344	>95† (N = 6)	1.50 ± 0.33‡ (N = 6)	5.48 ± 0.47 (N = 6)

Each value represents the mean ± SD. Note that the low dose of AL 1576 is equivalent to high doses of tolrestat and Ponalrestat in inhibiting sorbitol formation in red blood cells.
*The IC_{50} ratio of aldose reductase vs aldehyde reductase [23].
†Peak is within noise.
‡$P < 0.05$ vs control.
§Reference 22.

extent as the more specific aldose reductase inhibitors tolrestat and Ponalrestat, should be more effective at inhibiting the reduction of DHMAL to DHPG. On the other hand, if aldose reductase is important, no difference in the reduction of DHMAL to DHPG should be observed among the three inhibitors. The data were clear. There was no correlation between the selectivity of the inhibitor and the effect on NE metabolism when doses of inhibitors equipotent for blockade of sorbitol formation were compared. This strongly suggests that aldose reductase, rather than aldehyde reductase, is the primary enzyme responsible for the formation of DHPG under physiological conditions. These *in vivo* data again were consistent with the *in vitro* results.

Interestingly, the first recognizable change in NE metabolism induced by aldose reductase inhibitors was the increase of DHMA, but not the decrease of DHPG. The level of DHPG was maintained unchanged until the reduction pathway was blocked almost completely. This is because, under normal conditions, the production rate of DHPG is much higher than that of DHMA. Thus, small changes in the flux from reduction to oxidation pathways are more easily detected as a large relative increase of DHMA than the small decrease in DHPG [22].

Although aldose reductase is the primary enzyme responsible for DHPG formation, the much greater increases in DHMA and the significant fall in DHPG after the high dose of AL 1576 (when both aldose reductase and aldehyde reductase are inhibited) indicate that aldehyde reductase also may contribute to DHPG formation when aldose reductase is inhibited. Thus, not only is there redundancy in metabolism of catecholamines by MAO and catechol-O-methyltransferase, or in metabolism of aldehyde intermediates via oxidation and reduction pathways, but there also appears to exist redundancy of enzymes responsible for reduction of the aldehyde intermediates. However, this extra level of metabolic redundancy is perhaps not surprising, considering the potential toxicity of the aldehyde intermediates.

Based on the evidence that abnormal accumulation of sorbitol initiates various diabetic complications [26], worldwide efforts have been made to develop clinically potent aldose reductase inhibitors. An understanding of the redundant nature of NE metabolism may be important for the development of aldose reductase inhibitors without potential undesirable side-effects associated with increased levels of aldehyde intermediates.

Despite the overwhelming evidence for the adverse role of aldose reductase and the polyol (sorbitol) pathway in initiating diabetic complications, the physiological role of this enzyme and pathway in most tissues remains unknown. Similarly, the physiological substrate(s) of aldose reductase is generally unknown. An exception is the kidney, where sorbitol can serve as an osmolyte, and levels of aldose reductase in the inner medulla are regulated by osmolarity [27, 28]. Recently, a physiological role of aldose and aldehyde reductases in steroid metabolism has been suggested. The sequence of 20α-hydroxysteroid dehydrogenase also has been reported to be identical to that of aldose reductase [29]. Aldose reductase levels in rat ovary are regulated by the sexual cycles of the rat [30]. A number of steroids including 17α-hydroxyprogesterone can serve as substrates for aldose reductase with K_m values in the micromolar range [31]. The present study also establishes the role of aldose reductase in NE metabolism. Since the metabolism of NE to DHPG represents a major pathway for NE metabolism, this function of aldose reductase may be important.

It is also important to note that the distribution of aldo-keto reductases varies among species. Because rats generally contain high levels of aldose reductase, the present conclusions should be limited to this species. Further studies are required to confirm the importance of aldose reductase versus aldehyde reductase in NE metabolism in other species, including humans.

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References

1. Graefe K-H and Henseling M, Neuronal and extraneuronal uptake and metabolism of catecholamines. *Gen Pharmacol* **14**: 27–33, 1983.
2. Rutledge CO and Jonason J, Metabolic pathways of dopamine and norepinephrine in rabbit brain *in vitro*. *J Pharmacol Exp Ther* **157**: 493–502, 1967.
3. Kopin IJ, Catecholamine metabolism: Basic aspects and clinical significance. *Pharmacol Rev* **37**: 333–364, 1985.
4. Turner AJ and Tipton KF, The characterization of two reduced nicotinamide-adenine dinucleotide phosphate-linked aldehyde reductases from pig brain. *Biochem J* **130**: 765–772, 1972.
5. Tabakoff B, Anderson R and Alivisatos SGA, Enzymatic reduction of "biogenic" aldehydes in brain. *Mol Pharmacol* **9**: 428–437, 1972.
6. Wermuth B and Münch JDB, Reduction of biogenic aldehydes by aldehyde reductase and alcohol dehydrogenase from human liver. *Biochem Pharmacol* **28**: 1431–1433, 1979.
7. Wermuth B, Aldo-keto reductases. *Prog Clin Biol Res* **174:** 209–230, 1985.
8. Hoffman PL, Wermuth B and Wartburg JP, Human brain aldehyde reductases: Relationship to succinic semialdehyde reductase and aldose reductase. *J Neurochem* **35**: 354–366, 1980.
9. Wermuth B, Bürgisser H, Bohren K and von Wartburg J-P, Purification and characterization of human-brain aldose reductase. *Eur J Biochem* **127**: 279–284, 1982.
10. Whittle SR and Turner AJ, Differential sensitivity of aldehyde reductase isoenzymes to sodium valproate. *Biochim Biophys Acta* **657**: 94–105, 1981.
11. Jez JM, Bennett MJ, Schlegel BP, Lewis M and Penning TM, Comparative anatomy of the aldo-keto reductase superfamily. *Biochem J* **326**: 625–636, 1997.
12. Sato S, Rat kidney aldose reductase and aldehyde reductase and polyol production in rat kidney. *Am J Physiol* **236**: F799–F805, 1992.
13. Old SE, Sato S, Kador PF and Carper DA, *In vitro* expression of rat lens aldose reductase in *Escherichia coli*. *Proc Natl Acad Sci USA* **87**: 4942–4945, 1990.
14. Laemmli UK, Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature* **15**: 680–685, 1970.
15. Shiono T, Sato S, Reddy VN, Kador PF and Kinoshita JH, Rapid purification and properties of rat lens aldose reductase. *Prog Clin Biol Res* **232**: 317–324, 1987.
16. Sambrook J, Fritsch EF and Maniatis T, *Molecular Cloning: A Laboratory Manual*, 2nd Edn. Cold Spring Harbor Laboratory Press, Plainview, NY, 1989.
17. Church GM and Gilbert W, Genomic sequencing. *Proc Natl Acad Sci USA* **81**: 1991–1995, 1984.
18. Kobayashi A and Fujisawa S, Effect of L-carnitine on mitochondrial acyl CoA esters in the ischemic dog heart. *J Mol Cell Cardiol* **26**: 499–508, 1994.
19. Eisenhofer G, Goldstein DS, Stull R, Keiser HR, Sunderland T, Murphy DL and Kopin IJ, Simultaneous liquid-chromatographic determination of 3,4-dihydroxyphenylglycol, catecholamines, and dihydroxyphenylalanine in plasma and their responses to inhibition of monoamine oxidase. *Clin Chem* **32**: 2030–2033, 1986.
20. Berkowitz BA, Moriyama T, Fales HM, Byrd RA and Balaban RS, *In vivo* metabolism of 3-deoxy-3-fluoro-D-glucose. *J Biol Chem* **270**: 12417–12423, 1990.
21. Karino K, Kador PF, Berkowitz B and Balaban RS, ^{19}F-NMR quantitation of lens aldose reductase activity using 3-deoxy-3-fluoro-D-glucose. *J Biol Chem* **266**: 20970–20975, 1991.
22. Kawamura M, Kopin IJ, Kador PK, Sato S, Tjurmina O and Eisenhofer G, Effects of aldehyde/aldose reductase inhibition on neuronal metabolism of norepinephrine. *J Auton Nerv Syst* **66**: 145–148, 1997.
23. Sato S and Kador PF, Inhibition of aldehyde reductase by aldose reductase inhibitors. *Biochem Pharmacol* **40**: 1033–1042, 1990.
24. Burke WJ, Schmitt CA, Gillespie KN and Li SW, Norepinephrine transmitter metabolite is a selective cell death messenger in differentiated rat pheochromocytoma cells. *Brain Res* **722**: 232–235, 1996.
25. Bhatnagar A, Liu SQ, Das B, Ansari NH and Srivastava SK, Inhibition kinetics of human kidney aldose and aldehyde reductases by aldose reductase inhibitors. *Biochem Pharmacol* **39**: 1115–1124, 1990.
26. Kador PF, The role of aldose reductase in the development of diabetic complications. *Med Res Rev* **8**: 325–352, 1988.
27. Garcia-Perez A and Burg MB, Renal medullary organic osmolytes. *Physiol Rev* **71**: 1081–1115, 1991.
28. Burg MB, Kwon ED and Kultz D, Regulation of gene expression by hypertonicity. *Annu Rev Physiol* **59**: 437–455, 1997.
29. Warren JC, Murdock GL, Ma Y, Goodman SR and Zimmer WE, Molecular cloning of testicular 20α-hydroxysteroid dehydrogenase: Identity with aldose reductase. *Biochemistry* **32**: 1401–1406, 1993.
30. Iwata N, Hara S, Nishimura C, Takahashi M, Mukai T, Takayama M and Endo T, Hormonal regulation of aldose reductase in rat ovary during the estrous cycle. *Eur J Biochem* **235**: 444–448, 1996.
31. Petrash JM, Harter TM and Murdock GL, A potential role for aldose reductase in steroid metabolism. *Adv Exp Med Biol* **414**: 465–473, 1997.