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INHIBITION OF HUMAN LENS ALDOSE REDUCTASE BY FLAVONOIDS, SULINDAC AND INDOMETHACIN

PREM S. CHAUDHRY, JOSÉ CABRERA, HECTOR R. JULIANI and SHAMBHU D. VARMA*
Department of Ophthalmology, University of Maryland, School of Medicine, Baltimore, MD 21201, U.S.A.

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Abstract—The inhibition of human lens aldose reductase by flavonoids has been studied. Quercetin, the major pentahydroxyflavone, was observed to inhibit human lens aldose reductase by 50% at a concentration of 5×10^{-6} M. The inhibitory activity of its 3-*O*-glucoside was similar to that of the parent aglycon. Glycosidation with L-sugar (quercitrin and guaijaverin), however, inproved the inhibitory activity (the IC_{50} values being 1×10^{-6} M and 2.5×10^{-6} M respectively). The improvement in inhibitory activity with glycosidation with L-sugar was also apparent from the high inhibitory activity of myricitrin as compared to myricetin, although the improvement in this case of hexahydroxy flavone glycosidation was significantly less than in the case of penthahydroxy flavone glycosidation. The structure–activity relationship observed for human lens enzyme was similar to that reported previously for rat lens enzyme. Inhibitory activity on the whole however, was lower with human lens enzyme. Some known inhibitors of cyclo-oxygenase such as indomethacin, aspirin and sulindac also inhibited human lens aldose reductase. Thus, an inhibitor of one of the enzymes may actually inhibit both and, when administered, may exert mixed physiological effects.

The genesis of sugar cataracts in experimental animals has been proposed to be initiated by aldose reductase catalyzed synthesis of polyols [1] and a concomitant osmotic hydration of the tissue [2]. The most convincing evidence of this hypothesis came from studies where, in systemic and topical administration of aldose reductase, inhibitors attenuated the formation of cataracts in animals that were fed excessive galactose or were rendered diabetic by streptozotocin and alloxon [3–6]. More recent studies point out a possible usefulness of aldose reductase inhibitors in improving motor nerve conduction velocity in humans with diabetes [7]. The inhibition of aldose reductase has been achieved by various classes of generic compounds such as isoquinolines, xanthones, and chromonones. The largest number of inhibitors has been discovered in the class of chromones popularly known as bioflavonoids [8]. In initial experiments the inhibitory activities of these compounds were tested against rat lens aldose reductase. It is, nevertheless, desirable that such compounds be examined for their efficacy against human aldose reductase from various tissues. Some studies involving inhibition of human lens aldose reductase at the enzyme level, as well as the organ culture level, have appeared recently [9–11]. Such studies are of pharmacokinetic and nutritional importance. In this study, we have examined inhibition of purified human lens aldose reductase by certain bioflavonoid derivatives not reported before, such as glycosides and sulfate esters. Information has also been provided on the inhibition of human lens aldose reductase by indomethacin [1-(*p*-chlorobenzoyl)-5-methoxy-2-methylindole-3-acetic acid] and sulindac (cis-5-fluoro-2-methyl-1-*p*-(methylsulfinyl)benzylidene]indene-3-acetic acid), compounds recently reported to inhibit crude lens enzyme [10].

* Address correspondence to: Shambhu D. Varma, Ph.D., University of Maryland, School of Medicine, Department of Ophthalmology, 650 West Baltimore St, Baltimore MD 21201, U.S.A.

MATERIALS AND METHODS

Human lenses obtained from the Maryland eye bank within 4–6 hr after death were used for the enzyme preparation. The entire preparation of the enzyme was done in a cold room at 4°. Lenses were homogenized in 1.5 vol. (six lenses in 9 ml) of 50 mM phosphate buffer, pH 6.7, containing 10 mM mercaptoethanol. The homogenate was centrifuged at 12,000 *g* for 15 min to obtain the soluble enzyme fraction. The residue was re-extracted with one-third volume of the same buffer (3 ml). The two supernatant fractions were pooled and recentrifuged. Solid $(NH_4)_2SO_4$ was then added to the supernatant fraction to 25% saturation. After 15 min of equilibration, the protein that was precipitated was removed by centrifugation. Additional $(NH_4)_2SO_4$ was then added to the supernatant fraction to 60% saturation. This resulted in the precipitation of the enzyme activity. The precipitate thus obtained after centrifugation was dissolved in the minimum volume (3 ml) of the above buffer.

Gel filtration. The enzyme solution obtained as described above (ca 160 mg protein) was applied to a Sephadex G-75 column (1.5 × 85 cm) which had been equilibrated previously with 50 mM phosphate buffer, pH 6.7, containing 10 mM mercaptoethanol and 50 mM NaCl. The enzyme was eluted from the column with the same buffer in 2.5-ml fractions. Fractions with high enzyme activity were pooled and

used for further studies. The fractions collected between 61 and 73 ml were most active.

Enzyme activity was determined, as described previously, by the rate of decrease in $O.D._{340}$ due to NADPH utilization, monitored on a Gilford 2400-2 recording spectrophotometer [8]. The reaction mixture consisted of 2.5×10^{-4} M NADPH, 1.5×10^{-3} M DL-glyceraldehyde, in 0.1 M phosphate buffer, pH 6.7, and the enzyme in a final volume of 1 ml. The reference blank consisted of all the above components except the substrate. The effect of inhibitors on enzyme activity was determined by including in the mixture described above the compound under study at the desired concentrations. An appropriate blank to correct for absorption by the compounds was also run simultaneously. The compounds were dissolved in water with the aid of a minimum amount of sodium hydroxide which maintained the pH between 7 and 8. Indomethacin was dissolved in a minimum acount of ethanol.

During $(NH_4)_2SO_4$ fractionation and gel filtration, the activities of the fractions were monitored in the presence of 0.5 M lithium sulfate. Protein was determined according to Lowry *et al.* [12], using bovine serum albumin as the standard.

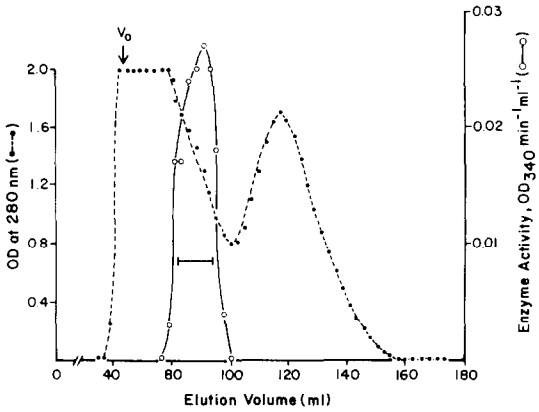

Fig. 1. Elution profile of aldose reductase on Sephadex G-75. Ammonium sulfate precipitate (about 160 mg protein containing the enzyme), dissolved in 3 ml of buffer, was applied to a 1.5×85 cm column. The column was pre-equilibrated by passing several column volumes of buffer. The fractions, 61–73 ml, having aldose reductase activity were pooled.

RESULTS AND DISCUSSIONS

The procedure described above yielded a preparation with a specific activity twenty time higher than that of the initial supernatant fraction. The elution pattern of aldose reductase through Sephadex G-75 is shown in Fig. 1. The enzyme activity was fairly stable under the given set of conditions, at least for 6 days at 4°. Thus, the preparation was quite suitable for testing the inhibitory activity of various compounds. With this preparation, no addition of lithium sulfate (often added to activate the enzyme if its specific activity is low) was required.

Sorbinil [*S*-6-fluoro-spiro-(chroman-4, 4-imidazolidine)-2, 5-dione] (Pfizer Chemical Co), and alrestatin (AY-22,284; 1, 3-dioxo-1*H*-benz de isoquionline-2 [3H] acetic acid) (Ayerst Laboratories, Montreal) already approved for clinical investigations, were used as reference compounds. The results with these and other compounds are described in Table 1. Sorbinil inhibited aldose reductase activity by 50% at 0.2×10^{-6} M. This IC_{50} value is close to the one previously reported for this enzyme preparation using lithium sulfate [9]. The IC_{50} for alrestatin was 6×10^{-6} M, a value approximately 3-fold higher than that previously reported with assays in the presence of lithium sulfate. Addition

Table 1. Inhibition of human lens aldose reductase by various compounds

Inhibitor*	Percent inhibition at the following concentrations					IC_{50} ($\times 10^{-6}$ M)
	10^{-5} M	10^{-6} M	10^{-7} M	10^{-8} M	10^{-9} M	
Sorbinil	100	68	39			0.2
Abrestatin (Ay 22,284)	62	36				6.0
Quercetin	59	32				5.0
Taxifolin	60	22				8.0
Quercetin-3-*O*-glucoside	42					
Quercetin-3-*O*-galactoside	50	37				10.0
Guaijaverin	78	37	15			2.5
Quercitrin	76	50	15			1.0
Acetyltrisulfate quercetin	100	85	50	42	12	0.1
Myricetin	47	26				
Myricitrin	74	50	12			1.0
Hexahydroxybenzophenone	59	40				5.0
Mono-7-hydroxyethyl quercetin	40	9				
6-Hydroxy-7-methoxy coumarin	36					
Indomethacin	34	20				
Sulindac	76	59	17			0.4
Sulindac sulfide	63	56	18			0.8
Aspirin	18					

* The flavanoids non-commercially available were obtained from H. R. Julian from the National University of Cardova, Argentina.

of lithium sulfate could, therefore, modify the response of the enzyme toward some inhibitors more strongly than others. The inhibition of aldose reductase by indomethacin, sulindac and its active metabolite (sulindac sulfide), which are commonly used anti-rheumatic drugs, was also studied. The IC_{50} values for sulindac and sulindac sulfide were 0.4×10^{-6} and 0.8×10^{-6} M respectively. The values are somewhat higher than those reported recently with crude lens enzyme [10].

We discovered previously that flavonoids are potent inhibitors of rat lens aldose reductase [13]. The main objective of the present investigation was to study the effect of some flavonoids on human lens aldose reductase. The IC_{50} of quercetin, the basic compound in this group, was 5×10^{-6} M. The IC_{50} of taxifolin (2,3-dihydroquercetin) was 8×10^{-6} M. The decreased electron density between the second and third carbon atoms of the benzo chromone moiety was thus associated with a loss in inhibitory potency. The nucleophilic character of the molecule was thus conducive to the inhibitory activity. A similar conclusion was drawn earlier on the basis of our previous studies conducted with the crude rat lens enzyme [8]. The most pronounced and interesting effect of structural modification of flavonoids with inhibitory ability was noticed in the glycoside group.

Quercetin-3-*O*-glucoside, with an inhibition of 42% at 10^{-5} M, quercetin-3-*O*-galactoside (IC_{50} of 10^{-5} M), and quercetin-3-*O*-L-arabinoside (IC_{50} of 2.5×10^{-6} M) were studied as representative compounds. The IC_{50} of the aglycon quercetin was 5×10^{-6} M. Thus, glycosidation with glucose as well as galactose both lowered the inhibitory activity slightly. Glycosidation of the 3-OH by an L-sugar, on the contrary, had a favorable effect. The inhibitory activities of both quercitrin (quercetin-3-*O*-L-rhamnoside) and guaijaverin (quercetin-3-*O*-L-arabinoside) were significantly higher than that of quercetin. They were also higher in comparison to those of the glucoside and the galactoside. Glycosidation with L-sugar, thus was definitely beneficial to the inhibitory potency of the flavanoids, glycosidation with an L-hexose (rhamnose) being more beneficial than with an L-pentose (arabinose). The IC_{50} of myricitrin, of the 3-*O*-L-rhamnoside of 3,5,7,3′,4′,5′-hexahydroxyflavone, was also observed to be higher than the corresponding aglycon; the IC_{50} for myricetin was similar to that of quercetin.

The most potent flavonoid in this series of investigations was the 3-*O*-acetyl-7,3,′4′-trisulfate of quercetin. It inhibited the enzyme activity by 42% at 10^{-8} M, and retained some inhibition at 10^{-9}; the IC_{50} was 0.1×10^{-6} M. This is very close to that observed for sorbinil, a compound that has been cleared for human trial. Other compounds tested had rather mild inhibitory activities. Although the results of our previous studies [8] with crude rat lens enzyme may not be comparable to the present results using human lens enzyme because of species difference, the results still suggest that a similar structure-activity relationship holds in most cases. An unsaturation of the chromone part of the molecule, glycosidation with L-sugars, and the presence of a ketone group are favorable features. Anthocyanins, where the C=O of flavone is reduced to —CH_2— were much less potent and hence not studied in detail. The high inhibitory activities of the sulfate esters point out the need of electronegative groups for inhibition, suggesting the possibility of an electrophilic nature of the enzyme center that is involved in enzyme–inhibitor interaction. The inhibition was of the non-competitive type (Fig. 2).

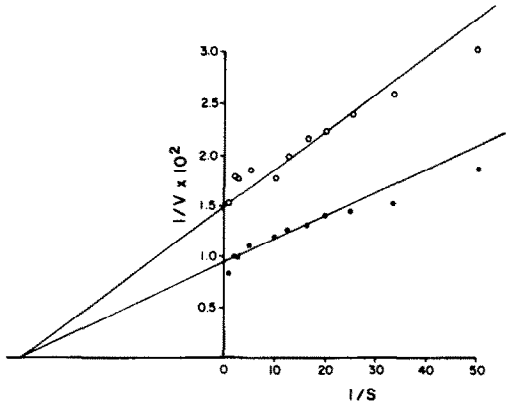

Fig. 2. Lineweaver–Burk plot of human lens aldose reductase. Key: (●—●) control, and (○—○) in the presence of 10^{-6} M quercitrin. The concentration of substrate, DL-glyceraldehyde, ranged from 0.02 to 1.0 mM. $V = O.D._{340} \cdot min^{-1} \cdot (mg\ protein)^{-1}$. The human lens aldose reductase had an apparent K_m of 0.025 mM, and querecitrin inhibited the enzyme activity non-competitively.

Some of the flavonoids such as quercetin have been shown to be mutagenic to certain strains of Salmonella. However, the mutagenicity is observed only when the compound is used along with dimethyl sulfoxide (DMSO) [14]. It is also known that not all compounds which induce mutation in bacterial cells will affect mammalian cells. Quercetin and its derivatives, which are present in our diet, are alluded to be such compounds [14]. More interestingly, recent studies have demonstrated clearly that quercetin and rutin are not carcinogenic [15]. In view of these recent developments and the emergence of more convincing evidence regarding the implications of aldose reductase in the genesis of human cataracts and neuropathy, the inhibition of human lens aldose reductase as well as that of other tissues by normal dietary constitutents is a very desirable goal. This is particularly true for the flavonoids because of their known nontoxicity despite long-term comsumption by humans. Further studies of the inhibition of human aldose reductase by commonly used dietary constituents and by drugs are in progress. Certain compounds that are inhibitory to aldose reductase may inhibit other enzyme systems as well. Flavonoids, for example, are known to inhibit phosphodiesterase [16]. They also have been suggested to be able to regulate the aberrant high glycolysis associated with certain tumor cells by inhibiting some species of Na^+-K^+-ATPase [17]. The other group of compounds which seems to have more than one mode of action appears to be represented by anti-inflammatory agents—sulindac, indomethacin and aspirin. These compounds which are known to inhibit prostaglandin synthesis [18], also inhibit aldose

reductase, as reported in this communication using purified human lens enzyme and as recently reported by another group [10] using crude lens homogenate. Ascertaining the inhibitory effect with purified enzyme obviously provided more definitive data. The multiplicity of the mode of action of certain compounds and the consequent diversity of systemic effects must, therefore, be considered when a selection is made to evaluate the pathogenesis of a disease or to conduct pharmacological studies. In the case of the eye, however, a large number of systemic effects can be curtailed by topical application instead of systemic administration of the drug in question. We, therefore, plan to evaluate the *in vivo* efficacy of inhibitors using various routes of administration as well as taking into consideration the multiplicity of action a particular compound may have.

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REFERENCES

1. R. van Heyningen, *Nature, Lond.* **184**, 194 (1959).
2. J. H. Kinoshita, *Invest. Ophthal.* **13**, 713 (1974).
3. D. Dvornik, N. Simmard-Dequesene, M. Krami, K. Sestang, K. H. Gabbay, J. H. Kinoshita, S. D. Varma and L. O. Merola, *Science* **182**, 1146 (1973).
4. S. D. Varma, A. Mizuno and J. H. Kinoshita, *Science* **195**, 205 (1977).
5. S. D. Varma and J. H. Kinoshita *Documenta Ophth.* **8**, 305 (1976).
6. L. T. Chylack, H. F. Henriques and W. H. Tung, *Documenta. Ophth.* **18**, 65 (1979).
7. R. Judzewitsh, J. B. Jaspan, M. A. Pfeifer, K. S. Polonsky, E. Halar, C. Vickadinovic, S. Richton, K. Gabbay, A. H. Richenstein and D. Porte, Jr., *Diabetes*, **30**, (Suppl. 1), (1981).
8. S. D. Varma and J. H. Kinoshita, *Biochem. Pharmac.* **25**, 2505 (1976).
9. F. Kador, J. H. Kinoshita, W. H. Tung and L. T. Chylack, Jr., *Invest. Ophthal. Vis. Sci.* **19**, 980 (1980).
10. Y. R. Sharma and E. Cotlier, *Expl. Eye Res.* **35**, 21 (1982).
11. S. D. Varma, S. S. Schocket and R. D. Richards, *Invest. Ophthal. Vis. Sci.* **18**, 237 (1979).
12. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
13. S. D. Varma, P. Mikuni and J. H. Kinoshita, *Science* **188**, 1215 (1975).
14. L. F. Bjeldanes and G. W. Chang, *Science* **197**, 577 (1977).
15. I. Hirono, I. Ueno, S. Hosaka, H. Takanashi, T. Matsushima, T. Sugimura and S. Natori, *Cancer Lett.* **13**, 15 (1981).
16. A. Beretz, M. Joly, J. C. Stoclet and R. Anton, *Planta med.* **36**, 193 (1979).
17. E. M. Soulina, D. R. Long and E. Racker, *J. natn. Cancer Inst.* **53**, 1515 (1974).
18. R. J. Flower, *Pharmac. Rev.* **26**, 33 (1974).