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Original Article

Effects of Anti-Inflammatory Drugs and ONO-2235 on Lens Aldose Reductase and on Sorbitol Accumulation in Red Blood Cells

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Abstract: Anti-inflammatory drugs and ONO-2235 were tested for their ability to inhibit lens aldose reductase and sorbitol accumulation in red blood cells. Sulindac, an anti-inflammatory drug, and ONO-2235 were shown to be potent inhibitors of aldose reductases from human cataractous lenses, bovine lenses and rat lenses. ONO-2235 was the most potent inhibitor with IC_{50} of 2×10^{-8} M, using human lens aldose reductase. Sulindac and ONO-2235 also inhibited the accumulation of sorbitol in human red blood cells. The IC_{50}s were 6.2×10^{-6} M, and 6×10^{-6} M, respectively.

Similarly, indomethacin and its prodrug, acemetacine, inhibited both aldose reductase in rat lens and sorbitol accumulation in human red blood cells. Furthermore, in diabetic rats, sulindac significantly suppressed sorbitol accumulation in red blood cells and sciatic nerves. These results suggest that sulindac as well as ONO-2235 can be useful in preventing chronic diabetic complications.

Key words: Anti-inflammatory drug, ONO-2235, Aldose reductase, Sorbitol, Diabetes mellitus

INTRODUCTION

Since the advent of insulin therapy for diabetics, one of the major concerns among diabetologists has been the prevention and treatment of chronic diabetic complications. A large number of reports have been made on the pathogenesis of diabetic complications[1]. Recently, the increased activity of the polyol pathway, which results in the accumulation of sorbitol, has been implicated in the pathogenesis of some chronic complications of diabetes[1,2]. Many publications have indicated that these complications related to the polyol pathway include cataracts[3-7], peripheral neuropathy[7-10], and more recently, retinopathy[11,12] and nephropathy[13]. It has also been shown that cataracts and peripheral neuropathy can be improved by the inhibitors of aldose reductase, the rate-limiting enzyme of the polyol pathway, in experimental animals[4-6] as well as in clinical trials[8].

In this report, we describe the effects of some anti-inflammatory drugs and ONO-2235, a new aldose reductase inhibitor, on lens aldose reductase and on sorbitol accumulation in red blood cells.

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MATERIALS AND METHODS

Preparation of crude aldose reductase from lenses

Human lenses were obtained from patients who had undergone cataract surgery. Bovine lenses were brought on ice from a local slaughterhouse. Rat lenses were obtained from 6-week-old male Wistar rats killed under ether anesthesia. All lenses were stored at −20°C until use. Lenses were homogenized in a 135 mM Na, K-phosphate buffer (pH 7.0) containing 0.5 mM phenylmethyl sulfonyl fluoride (PMSF) and 10 mM 2-mercapto-ethanol, and then centrifuged at 100,000×*g* for 30 min. The supernatants were used as enzyme fractions. All the procedures were carried out at 4°C.

Aldose reductase activities

Aldose reductase activity was assayed according to the method previously described by Dufrane *et al.*[14], with minor modifications[15]. The incubation mixture contained 135 mM Na, K-phosphate buffer (pH 7.0), 100 mM $LiSO_4$, 0.03 mM NADPH, 0.1 mM DL-glyceraldehyde or 20 mM glucose and 100 μl of enzyme fractions with or without 100 μl of various concentrations of aldose reductase inhibitors in a total volume of 1.0 ml. The inhibitors were dissolved in 1% ethanol. The control preparations contained 100 μl of 1% ethanol to make a final concentration of ethanol of 0.1%. The reaction was initiated by the addition of NADPH at 30°C and stopped by adding 0.3 ml of 0.5 N HCl, and then 1 ml of 6 N NaOH containing 10 mM imidazole was added to fluoresce NADP. The fluorescence was measured at room temperature by Hitachi fluorescence spectrophotometer F-3000 (Hitachi, Japan) with an excitation wave length of 360 nm and an emission wave length of 460 nm. Standards of NADP (50–2000 pmoles/tube) were also treated with 0.3 ml of 0.5 N HCl, followed by the addition of 1 ml of 6 N NaOH containing 10 mM imidazole[16].

Protein concentrations were determined by the method of Lowry *et al.*[17]

Sorbitol contents in red blood cells

Red cells from heparinized healthy human blood were washed three times with cold saline by centrifugation (2,000 × *g*, 5 min) at 4°C and resuspension. One milliliter of the human red blood cell suspension was incubated in 4 ml of medium equilibrated with 95% O_2–5% CO_2 at 37°C. The medium used in this study was Krebs-Ringer bicarbonate buffer (pH 7.4) containing 28 mM glucose and 50 μl of various concentrations of aldose reductase inhibitors. After 60 min incubation, red blood cells were quickly washed three times with cold saline, and precipitated with 6% cold perchloric acid. They were then centrifuged again, and the supernatant was neutralized by adding 1/10th the volume of 2.5 M K_2CO_3. The sorbitol concentration was determined by the method of Malone *et al.*[15,18]

Diabetic rats

Diabetes was induced in male Wistar rats (120–160 g) by a single intraperitoneal injection of streptozotocin (80 mg/kg) in sodium citrate buffer, pH 4.5. Seven days after the induction of diabetes, animals were given sulindac or vehicle alone via gastric tubing, twice a day, in doses of 10, 20 or 40 mg/kg/day for 4 weeks. Sulindac was suspended in saline containing 0.5% carboxy-methyl cellulose. The animals were then killed under ether anesthesia. The content of sorbitol in rat red blood cells were determined by the same method without incubating in Krebs-Ringer bicarbonate buffer. Sciatic nerves and lenses were quickly removed by incision, weighed and homogenized in distilled water and then treated in the same manner as the red blood cells.

Sources of materials

Sulindac was provided by Banyu Pharmaceutical Co., Ltd. (Tokyo Japan), indomethacin and acemetacine by Kowa

Pharmaceutical Co., Ltd. (Tokyo, Japan), phenytoin by Dainippon Pharmaceutical Co., Ltd. (Tokyo, Japan) and carbamazepine by Ciba-Geigy Japan Co., Ltd. (Takarazuka, Japan). ONO-2235, {(E)-3-carboxymethyl-5-[(2E)-methyl-3-phenyl-propenylidene] rhodanine}, was generously donated by Ono Pharmaceutical Co., Ltd. (Osaka, Japan). NAD, NADP, NADPH and sorbitol dehydrogenase were purchased from Boehringer Mannheim Co. (Mannheim, West Germany). PMSF, $LiSCO_4$ and streptozotocin were obtained from Sigma (St. Louis, Missouri). Six-week-old male Wister rats were obtained from Shizuoka Experimental Animal Co., Ltd. (Hamamatsu, Japan).

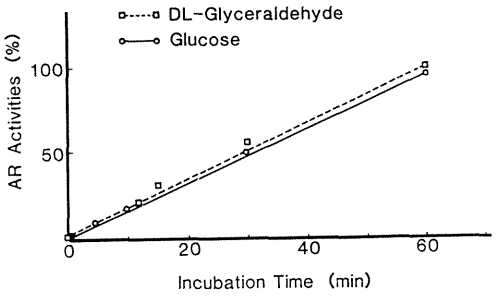

Fig. 1. Time course of AR activity with RLAR as the enzyme fraction. The abscissa represents incubation time and the ordinate, AR activity. The enzyme activities at each incubation time were expressed as relative activities compared with that at 60 min of incubation with DL-glyceraldehyde or glucose as a substrate. The slopes were derived using a least-square regression analysis.

Results

Time course of aldose reductase activities

The activity of rat lens aldose reductase (RLAR) was almost linear during the 60 min incubation period (Fig. 1). Similar results were also obtained with human (HLAR) and bovine lens aldose reductase (BLAR) (data not shown). In the remainder of the experiments, therefore, the incubation periods were set at 30 min. Table 1 summarizes the specific activities and Km of these enzymes for DL-glyceraldehyde or glucose. Among them, RLAR showed the highest specific activity, about 16 times that of HLAR and BLAR, when DL-glyceraldehyde was used as a substrate. On the other hand, Km for either DL-glyceraldehyde or glucose did not show any significant differences between these enzyme sources.

Effects of various concentrations of sulindac, ONO-2235, phenytoin and carbamazepine on HLAR, BLAR and RLAR.

Sulindac and ONO-2235 markedly inhibited aldose reductase activities in a dose-related manner (Fig. 2). Since phenytoin and carbamazepine were known to be effective in treating diabetic neuropathy, they were also tested for their ability to inhibit aldose reductase activities. HLAR activity, however, was not detected enough to recognize the inhibition by these drugs when glucose was used. Compared to sulindac and ONO-2235, phenytoin required much higher concentrations to elicit an appreciable inhibition. Carbamazepine showed no

Table 1. Specific Activities and Km of Aldose Reductases

	Specific Activities (U)*		Km (mM)	
Substrate	DL-glyceraldehyde	Glucose	DL-glyceraldehyde	Glucose
HLAR	109	5	0.027	—
BLAR	116	23	0.011	29
RLAR	1690	534	0.045	66

*: One unit of aldose reductase activity indicates the activity that converts 1 pmole of NADPH to NADP per mg protein per minute.

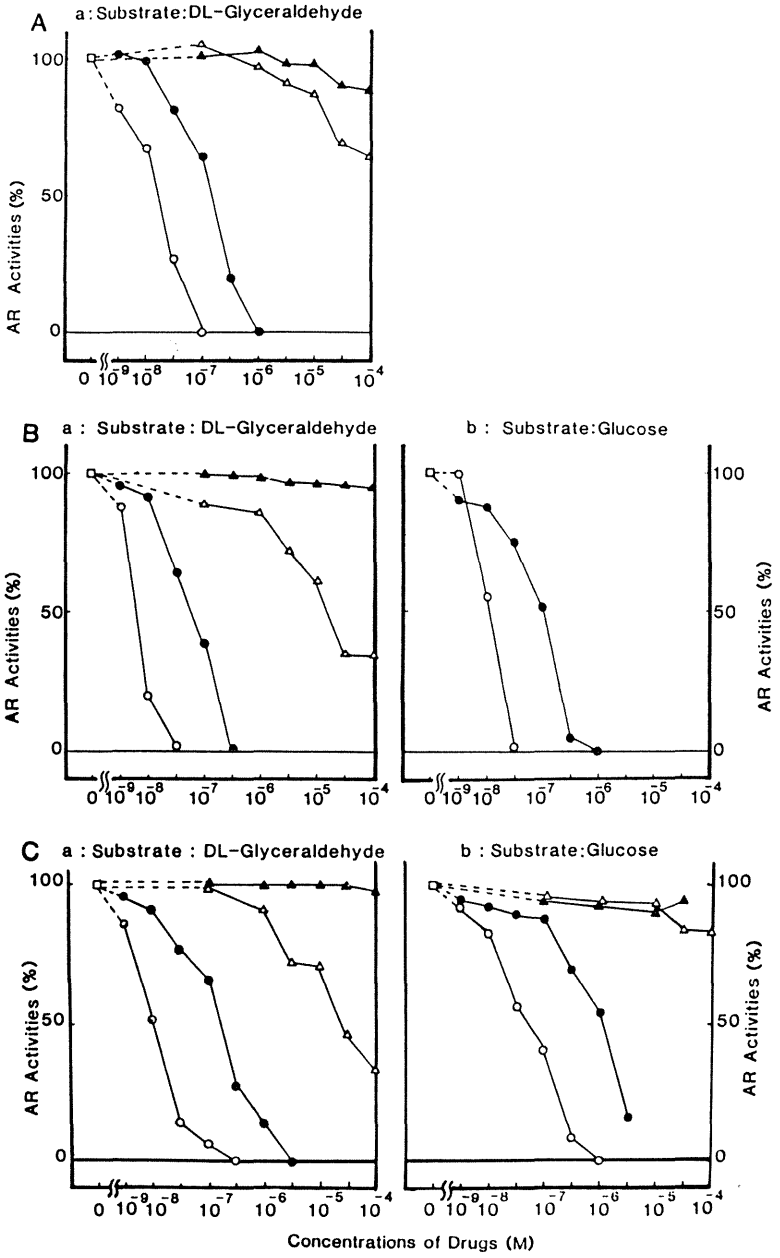

Fig. 2. Inhibition of AR activity by various inhibitors, with HLAR (A) BLAR (B), or RLAR (C) as the enzyme. The abscissa represents the concentrations of drugs, and the ordinate the activity of AR relative to its activity in the absence of a drug. Inhibitors used were ONO-2235 (○), sulindac (●), phenytoin (△), and carbamazepine (▲). Substrates used were DL-glyceraldehyde (a) and glucose (b).

significant inhibition even at a concentration of 10^{-4} M. Table 2 summarizes inhibiting potencies of aldose reductase by these drugs in terms of IC_{50}. Thus, ONO-2235 was consistently about 10–20 times more potent than sulindac under the experimental conditions.

Effects of indomethacin and acemetacine on RLAR

The effects of these two agents were studied only on RLAR since the relative activities as AR inhibitors were almost identical irrespective of the sources of enzymes. The two drugs markedly inhibited RLAR activity in a dose-dependent manner (Fig. 3). IC_{50}s of indomethacin and acemetacine were 1.7×10^{-6} M and 2.7×10^{-6} M, respectively (Table 2).

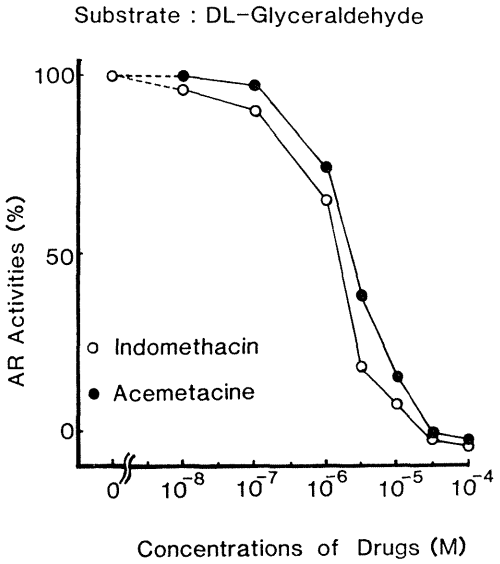

Fig. 3. Inhibition of RLAR activity by indomethacin and acemetacine, with DL-glyceraldehyde as a substrate.

Kinetic studies on the effects of sulindac and ONO-2235 on HLAR and RLAR with DL-glyceraldehyde as a substrate

When HLAR was used, the results with sulindac and ONO-2235 yielded roughly parallel slopes, which are characteristic of uncompetitive inhibition on Lineweaver-Burk plots. Increased concentrations of sulindac and ONO-2235 resulted in a convergence of the Kms in the reciprocal plot, indicating a non-competitive type of inhibition (Fig. 4). When RLAR was used, double reciprocal plots showed only a slight increase in the intercept on the ordinate with lower concentrations of sulindac and ONO-2235. Higher concentrations, however, caused movements of the intercepts on both the ordinate and the abscissa, indicating a non-competitive type of inhibition.

Effects of aldose reductase inhibitors on the accumulation of sorbitol in human red blood cells

Sulindac, indomethacin, acemetacine and ONO-2235 inhibited sorbitol accumulation in a dose-dependent manner (Fig. 5). On

Table 2. IC_{50}s of Various Drugs on Aldose Reductase Activities*

	Substrate	ONO-2235	Sulindac	Phenytoin	Carbamazepin	Indomethacin	Acemetacine
HLAR	DL-glyceraldehyde	2×10^{-8}	1.7×10^{-7}	—	—	N. T.	N. T.
	Glucose	N. T.	N. T.	N. T.	N. T.	N. T.	N. T.
BLAR	DL-glyceraldehyde	0.36×10^{-8}	7.5×10^{-8}	1.9×10^{-5}	—	N. T.	N. T.
	Glucose	0.7×10^{-8}	1.1×10^{-7}	N. T.	N. T.	N. T.	N. T.
RLAR	DL-glyceraldehyde	0.9×10^{-8}	1.9×10^{-7}	3.6×10^{-5}	—	1.7×10^{-6}	2.7×10^{-6}
	Glucose	6.5×10^{-8}	1.2×10^{-6}	—	—	N. T.	N. T.

*: Concentrations were expressed in M.
N. T.: not tested

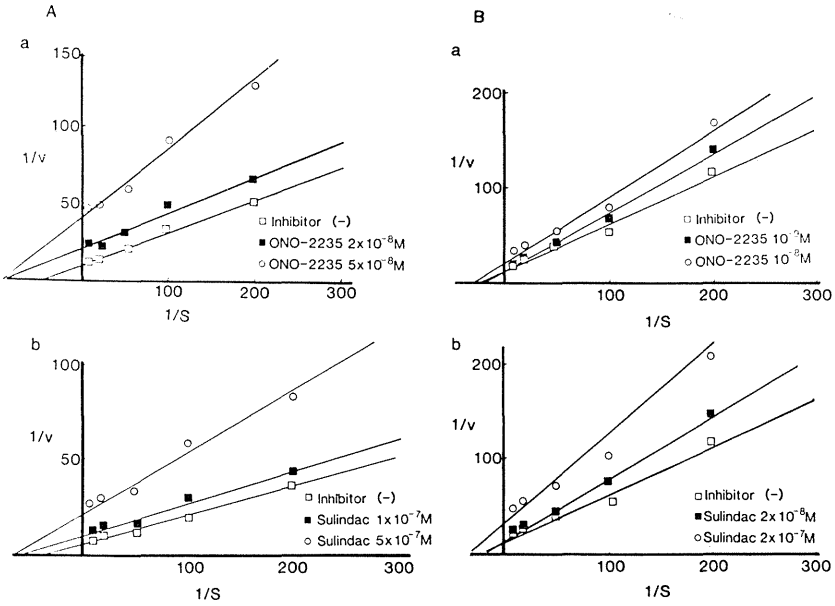

Fig. 4. Double reciprocal plots of HLAR (A) and RLAR (B) activity with DL-glyceraldehyde as a substrate. The abscissa represents the reciprocal of DL-glyceraldehyde concentration between 5×10^{-6} M and 2×10^{-4} M. The ordinate represents the reciprocal of AR activities. Inhibitors tested were ONO-2235 (a) and sulindac (b). The slopes were derived using a least-square regression analysis.

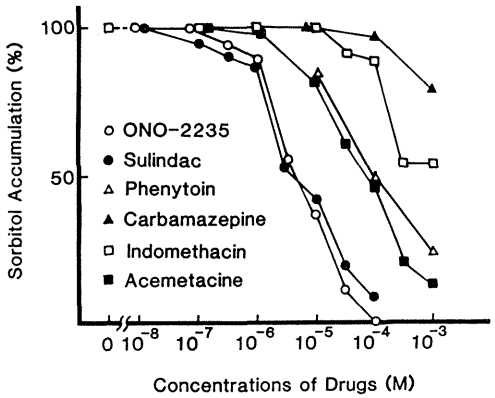

Fig. 5. Inhibition of sorbitol accumulation by AR inhibitors in human red blood cells. The abscissa represents the concentrations of inhibitors and the ordinate the accumulation of sorbitol as a percentage of the control value (without any inhibitors).

the other hand, phenytoin and carbamazepine needed much higher concentrations to obtain a significant suppression of sorbitol accumulation. The IC_{50}s of sulindac, indomethacin, acemetacine, phenytoin and ONO-2235 were 6×10^{-6} M, 7.0×10^{-4} M, 9.2×10^{-5} M, 0.9×10^{-4} M, and 6.2×10^{-6} M, respectively.

In vivo effects of sulindac on tissue sorbitol contents in diabetic rats

An *in vivo* study was also conducted to evaluate the effects of sulindac on sorbitol accumulation in tissues of diabetic rats. There were no significant differences in body weights (141–156 g) and blood glucose concentrations (408–438 mg/dl) between the rats treated with sulindac and the diabetic control rats. In diabetic control rats, sorbitol contents in red blood cells (167.6± 15.4 nmol/g Hb) were much higher than those in normal rats (54.4±4.0) ($p<0.001$) (Fig. 6, A). In sulindac-treated rats, sorbitol contents in red blood cells were decreased dose-dependently. Sulindac also

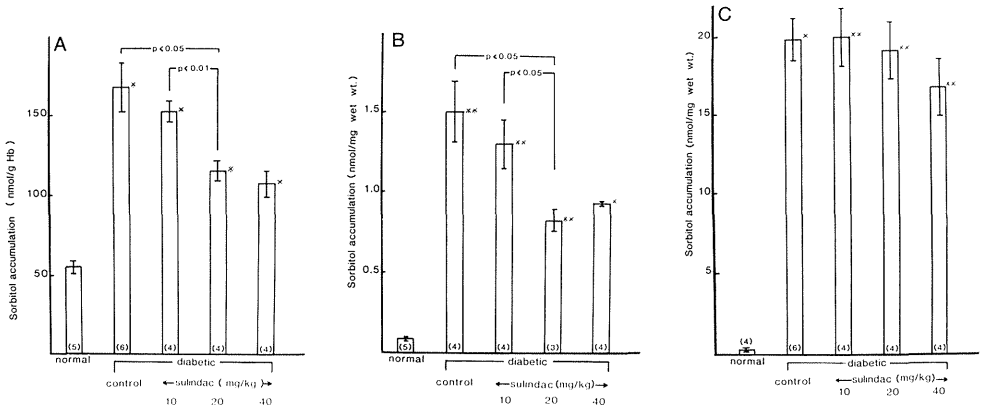

Fig. 6. The effect of sulindac on the accumulation of sorbitol in tissues of diabetic rats. A: red blood cells, B: sciatic nerve, C: lens. Animals were given the vehicle alone or sulindac by gastric tubing, twice daily at doses of 10, 20, or 40 mg/kg/day for 4 weeks. The number of animals in each group is given in parenthesis. The standard error of the means is illustrated by the vertical line. *: $P<0.001$ vs normal. **: $P<0.01$ vs normal.

suppressed sorbitol accumulation in sciatic nerves as shown in Fig. 6, B. In contrast, the sorbitol accumulation in lenses was not significantly inhibited by sulindac (Fig. 6, C).

Discussion

The Km values of HLAR and BLAR to DL-glyceraldehyde reported in literature to date, range from 0.024–0.1 mM[3,19,20] and 0.03–0.6 mM[21,22], respectively. Our results concerning the Km of HLAR (0.027 mM) is similar to those reported by Chaudhry *et al.*[18], who used purified enzymes. Among the drugs studied, ONO-2235 showed the most potent inhibition of HLAR, BLAR and RLAR[23] with IC_{50}s of 2×10^{-8}, 0.36×10^{-8} and 0.9×10^{-8} M, respectively, when DL-glyceraldehyde was used as a substrate. The IC_{50} of sulindac with HLAR was 1.7×10^{-7} M which is comparable with that of a previous report[19]. Various aldose reductase inhibitors such as flavonoids[19,24], anti-rheumatic drugs[20], sorbinil[7,25] and ONO-2235[22] have been studied. Varma and Kinoshita[26] reported that the type of inhibition of alrestatin and quercitrin is non-competitive, while Peterson *et al.*[27] reported that of sorbinil to be uncompetitive. Recently, Terashima *et al.*[23] and Kador and Sharpless[28] have reported that ONO-2235 shows either uncompetitive or non-competitive inhibition depending on the concentration. The results of the kinetic studies of sulindac and ONO-2235 showed both uncompetitive and non-competitive types of inhibition of HLAR. On the other hand, when RLAR was used, the lower concentrations of the inhibitors yielded somewhat ambiguous kinetic effects. Increasing the concentrations of the inhibitor, however, revealed that an apparent effects on the kinetics is non-competitive type of inhibition.

Sulindac, indomethacin, acemetacine and ONO-2235 inhibited sorbitol accumulation within human red blood cells. Compared with their effects on aldose reductase activities, however, much higher concentrations were required to achieve 50% inhibition of sorbitol accumulation. Sulindac was almost as potent as ONO-2235 in suppressing sorbitol accumulation, whereas the effectiveness of the former was about 1/10

that of the latter in inhibiting AR activity. The reason for this difference in effectiveness is not clear at present. A possible explanation may be that sulindac penetrates red blood cell membranes more readily than does ONO-2235. As for indomethacin, acemetacine, phenytoin and carbamazepine, much higher concentrations were required to elicit significant inhibitions of sorbitol accumulation within human red blood cells. Phenytoin and carbamazepine have been reported to improve the subjective symptoms of diabetic neuropathy[29,30]. It has also been reported that effective plasma concentrations of phenytoin and carbamazepine are approximately 40–80 μM[31] and 20–40 μM[32], respectively. Acemetacine is rapidly and almost completely metabolized to indomethacin in liver. The effective plasma concentration range of indomethacin was reported to be greater than 0.05–0.3 μg/ml[33] and less than 1 μg/ml (2.8×10^{-6} M)[34]. The mechanism of the effectiveness of phenytoin, therefore, can be partly explained by its inhibition of AR activity, while the other three agents probably do not involve the effects on the polyol pathway.

Cotlier *et al.*[35] reported that the galactose-fed rats developed cataracts at a slower rate when injected with sulindac compared with paired-fed saline-injected control rats. In diabetic patients receiving oral sulindac (200 mg twice daily), there were fewer alterations of the blood-retinal barrier which occur in the early stages of diabetic retinopathy, compared with the placebo-treated group during the six-month study[36]. On the contrary, Crabbe *et al.*[37] concluded that oral administration of sulindac results in concentrations in red blood cells which are too low to modify the polyol pathway. We disagree with their conclusion for the following reasons: First of all, in their study, the plasma concentration of sulindac (5.43 ± 3.5 μg/ml) was higher than that reflected by the IC_{50} data in this study regarding the inhibition of sorbitol accumulation in human red blood cells. Secondly, assuming that the IC_{50} of sulindac for human erythrocyte AR is comparable with that for HLAR, the concentration of sulindac within red blood cells that they have reported (0.17 ± 0.21 μg/ml) should be high enough to inhibit AR activity. Therefore, it is likely that usual oral doses of sulindac inhibits AR activity at least in red blood cells.

To confirm this hypothesis, we have studied the effect of sulindac on tissue sorbitol accumulation in diabetic rats. Sulindac significantly suppressed sorbitol accumulation in red blood cells and in sciatic nerves, but not in lenses. Since the accumulation of sorbitol is implicated in the pathogenesis of chronic diabetic complications, AR inhibitors can be useful in preventing and even improving these complications. Finally, our results suggest that sulindac as well as ONO-2235 is effective in preventing diabetic complications.

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