

# Aldose Reductase in Glucose Toxicity: A Potential Target for the Prevention of Diabetic Complications

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## I. Introduction

Recent data obtained from the Diabetes Control and Complications Trial clearly indicate that intensive insulin treatment effectively delays the onset and slows the progression of longterm diabetic complications in patients with insulin-dependent diabetes mellitus (IDDM)<sup>b</sup> (Diabetes Control and Complications Trial Research Group, 1993). Nevertheless, even with the best clinical management available at present, it is practically impossible to maintain normoglycemia at all times throughout the life of diabetic individuals. Accordingly, chemical agents that effectively halt the hyperglycemic injury in diabetic patients would be of great clinical importance.

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<sup>b</sup> Abbreviations: cDNA, complementary deoxyribonucleic acid; DAG, diacylglycerol; FR-1, fibroblast-growth-factor-1-regulated protein; HbA<sub>1c</sub>, glycosylated hemoglobin; IDDM, insulin-dependent diabetes mellitus; mRNA, messenger ribonucleic acid; MVDP, mouse vas deferens protein; NAD<sup>+</sup>, nicotinamide adenine dinucleotide; NADPH, reduced nicotinamide adenine dinucleotide phosphate; NIDDM, non-insulin-dependent diabetes mellitus; NO, nitric oxide.

Under normoglycemia, most of the cellular glucose is phosphorylated into glucose 6-phosphate by hexokinase. A minor part of nonphosphorylated glucose enters the so-called polyol pathway, the alternate route of glucose metabolism. The rate-limiting step of this polyol pathway is the reduction of glucose to sorbitol catalyzed by aldose reductase (EC 1.1.1.21). Sorbitol is subsequently converted to fructose by sorbitol dehydrogenase, thus constituting the polyol (sorbitol) pathway (fig. 1). Under hyperglycemia, because of the saturation of hexokinase with ambient glucose, the increased flux of glucose through the polyol pathway accounts for as much as one-third of the total glucose turnover (González et al., 1984). This leads to overflow of the products of the polyol pathway along with depletion in reduced nicotinamide adenine dinucleotide phosphate (NADPH) and the oxidized form of nicotinamide adenine dinucleotide (NAD<sup>+</sup>), the cofactors used in the pathway. The acceleration of the polyol pathway thus elicits various metabolic imbalances in those tissues that undergo insulin-independent uptake of glucose. Such metabolic perturbation provokes the early tissue damage in the "target" organs of diabetic complications, such as ocular

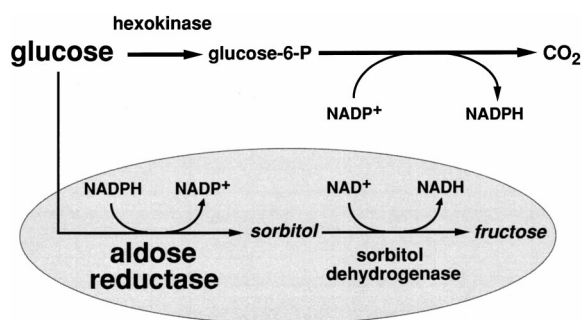


FIG. 1. Polyol (sorbitol) pathway; glucose-6-P, glucose 6-phosphate.

lens, retina, peripheral nerve, and renal glomerulus (Kinoshita and Nishimura, 1988; Pugliese et al., 1991).

These observations led to the development of numerous aldose reductase inhibitors of diverse chemical structures as possible therapeutic agents for diabetic complications. Sorbinil, ponalrestat, and tolrestat were among the most studied inhibitors to prevent cataracts, retinal capillary basement membrane thickening, and nerve conduction velocity deficits in experimental diabetic animal models. Until now, however, the clinical efficacy of these inhibitors in diabetic patients has not been fully proved to meet the standards of the Food and Drug Administration (Pfeifer et al., 1996).

In this review, recent advances in the understanding of the pathophysiological significance of aldose reductase are presented that would be relevant to the efficacy of the enzyme inhibitors in clinical intervention trials of diabetic complications. An extensive review on the pathogenesis of diabetic complications is outside the scope of this review. For this topic, the reader is encouraged to refer to other excellent reviews published elsewhere (Pugliese et al., 1991; Greene et al., 1993; Cameron and Cotter, 1994; Yagihashi, 1995).

## II. Aldose Reductase and Aldo-Keto Reductase Superfamily

### A. Aldose Reductase as a Member of the Aldo-Keto Reductase Superfamily

Aldose reductase is a small monomeric protein composed of 315 amino acid residues. The primary structure, first determined on rat lens aldose reductase (Carper et al., 1987, 1989), demonstrated high similarities to another NADPH-dependent oxidoreductase, human liver aldehyde reductase (EC 1.1.1.2) (Wermuth et al., 1987) and to  $\rho$ -crystallin, a major structural component of the lens of frog *Rana pipiens* (Tamarev et al., 1984). The degree of similarity clearly suggests that these proteins belong to the same family, namely aldo-keto reductase superfamily, with related structures and evolutionary origins. Subsequently, complementary deoxyribonucleic acid (cDNA) clones of human placenta, retina, and muscle aldose reductase were isolated (Bohren et al., 1989; Chung and LaMendola, 1989; Nishimura et al., 1990). As the coding sequences of these

cDNAs originating from different tissues turned out to be essentially identical, the existence of tissue-specific isoforms for human aldose reductase has yet to be verified. The identification of amino acid sequences of aldose reductase from different species revealed a relatively low sequence identity (82–85%) conserved among human, rat, and other animal species. This could account for the species' differences in the sensitivity of aldose reductase to some of the inhibitors. For screening the potency and efficacy of the newly designed inhibitors, human enzyme preparations may be preferable and can be readily obtained by the recent recombinant technique (Nishimura et al., 1991).

In the past few years, molecular cloning techniques have identified many amino acid sequences for cellular proteins and a recent search of a database of these sequences identified as many as 39 proteins as members of the aldo-keto reductase superfamily (Jez et al., 1996). A wide variety of proteins from various species constitute this family, including aldehyde and xylose reductases from plants, yeast, and bacteria, as well as bovine and rat *Shaker*  $K^+$  channel  $\beta$ -subunit (Rettig et al., 1994). Nonetheless, the majority of this family is represented by mammalian aldehyde reductases, aldose reductases, and hydroxysteroid dehydrogenases. Approximately 50% of the amino acid sequences are conserved between aldose reductase and these mammalian enzymes from various species and tissues. For instance, among the members coexisting in human liver are aldehyde reductase (Bohren et al., 1989), hepatic bile acid-binding protein (Stolz et al., 1993),  $\Delta^4$ -3-oxosteroid  $5\beta$ -reductase (Kondo et al., 1994), type I  $3\alpha$ -hydroxysteroid dehydrogenase, also referred to as chlordecone reductase (Winters et al., 1990), and type II  $3\alpha$ -hydroxysteroid dehydrogenase (Khanna et al., 1995).

### B. A Closely Related Subgroup in the Aldo-Keto Reductase Superfamily

Interestingly, a unique subgroup was recently demonstrated in this superfamily. Cloning and determination of the amino acid sequence of mouse aldose reductase cDNA revealed the presence of closely related proteins with high sequence similarities in mouse (Gui et al., 1995). These are mouse vas deferens protein (MVDP) (Pailhoux et al., 1990) and fibroblast-growth-factor-1-regulated protein (FR-1) (Donohue et al., 1994), that respectively manifest as much as 69% identity to mouse aldose reductase. The identity between MVDP and FR-1 is nearly 82%. These three murine proteins therefore constitute a new subgroup within the aldo-keto reductase superfamily (fig. 2). Between rat and mouse aldose reductases, approximately 97% of the amino acid sequence is conserved. Analogously, one of the tumor-associated variants of protein detected in rat liver exhibited high sequence similarity to rat aldose reductase and to MVDP (Zeindl-Eberhart et al., 1994). This protein variant possibly belongs to the above subgroup of the

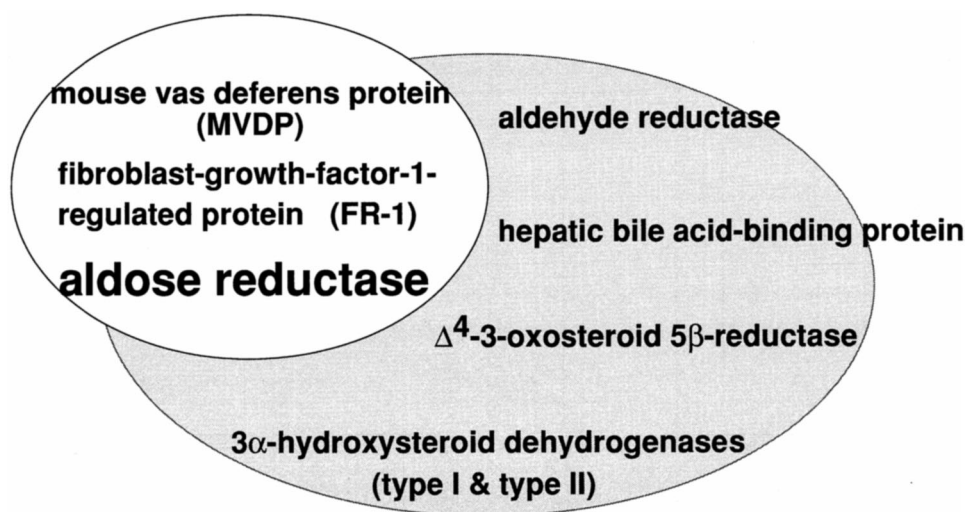


FIG. 2. Members of aldo-keto reductase superfamily identified in human and a closely related subfamily demonstrated in mouse (MVDP and FR-1).

aldo-keto reductase family as well. In view of these findings, the existence of hitherto unidentified proteins closely related to aldose reductase in humans can be speculated.

As the enzyme inhibitor zopolrestat was shown to bind and inhibit the action of mouse FR-1 to catalyze the reduction of DL-glyceraldehyde (Wilson et al., 1995), it is conceivable that the human counterpart of such related proteins may bind and prevent the action of inhibitors of aldose reductase. In fact, some aldose reductase inhibitors are known to equally interact with aldehyde reductase, a member of the aldo-keto reductase family that coexists with aldose reductase in most tissues (Sato and Kador, 1990). A widely used inhibitor like sorbinil inhibits aldose and aldehyde reductases with comparable  $IC_{50}$  values, whereas zopolrestat has higher specificity for aldose reductase as compared with aldehyde reductase (Barski et al., 1995).

### C. Tertiary Structure of Aldose Reductase

Crystallographic structures have been determined for pig (Rondeau et al., 1992) and human aldose reductases (Borhani et al., 1992; Wilson et al., 1992). The enzyme molecule contains a  $(\beta/\alpha)_8$  barrel structural motif with a large hydrophobic active site. The cofactor NADPH binds in an extended conformation to the bottom of the active site, located at the center of the barrel. The holoenzyme structure complexed with the enzyme inhibitor zopolrestat further demonstrated that the inhibitor binds to the active site on top of the nicotinamide ring of the NADPH (Wilson et al., 1993). When zopolrestat was complexed with the holoenzyme, however, it perturbed the position of two loops in the protein and changed the shape of the active site pocket. When the enzyme was complexed with another inhibitor sorbinil, the inhibitor simply occupied the active site pocket and did not induce further conformational change in the enzyme molecule (Urzhumtsev et al., 1997). These findings suggest that

many compounds with diverse chemical structures can interact with the enzyme in different conformations. This illustrates the dangers of using theoretical approaches to predict the rigid inhibitor binding site of aldose reductase, as the enzyme apparently retained considerable flexibility in its tertiary structure (Wilson et al., 1996).

As mentioned above, there are marked differences in the selectivity toward aldose and aldehyde reductases among the known enzyme inhibitors. Such selectivity has been attributed to the interaction of the inhibitors with the enzyme molecule, depending upon whether the enzyme opens a 'specificity' pocket. This pocket binds inhibitors that are more effective against aldose reductase than against aldehyde reductase (Urzhumtsev et al., 1997). In this regard, identification and characterization of as yet unknown members of the aldo-keto reductase subfamily in humans seem essential. To effectively block the enhanced flux of glucose through polyol pathway, the inhibitor needs to be specific for aldose reductase and devoid of intercalating into other structurally related proteins coexisting in the "target" organs of diabetic complications.

## III. Physiological Significance of Aldose Reductase

### A. Polyol Pathway First Identified in the Seminal Vesicle

At present, physiological functions of aldose reductase have not been entirely clarified. Aldose reductase is a cytosolic enzyme present in most of the mammalian cells, although the distribution of the enzyme is not uniform among tissues. Nearly forty years ago the polyol pathway was first identified in the seminal vesicle by Hers (Hers, 1956), who demonstrated the conversion of blood glucose into fructose, an energy source of sperm cells. Later Van Heyningen reported the presence of

sorbitol in diabetic rat lens (Van Heyningen, 1959), and her work provided an opening for new research concerning the pathological role of aldose reductase and the polyol pathway in the development of diabetic complications. In fact aldose reductase messenger ribonucleic acid (mRNA) in rat was highly expressed in the lens, the retina, and the sciatic nerve, the major "target" organs of diabetic complications (Nishimura et al., 1988). Ever since, inhibitors for aldose reductase have been expected to become a potential treatment modality in diabetes. However, it is necessary to understand the physiological relevance of the polyol pathway in view of the possible side effects arising from a prolonged inhibition of aldose reductase in diabetic patients.

### B. Osmoregulatory Role in the Kidney

In the previous decade, elevated extracellular NaCl was demonstrated to elicit marked increase in aldose reductase expression and accumulation of intracellular sorbitol in the cultured cell line from rabbit renal papilla (Bagnasco et al., 1987). In the kidney, aldose reductase mRNA was abundantly expressed in the medulla compared with relatively low expression in the cortex (Nishimura et al., 1988). These findings were confirmed by biochemical and immunohistochemical analyses of rat and human kidneys (Terubayashi et al., 1989). Sorbitol is one of the organic osmolytes that balance the osmotic pressure of extracellular NaCl, fluctuating in accord with urine osmolality (Burg, 1995). These findings, therefore, suggest the osmoregulatory role of aldose reductase in the renal homeostasis. The increased expression of aldose reductase under hyperosmotic stress was subsequently reported in a variety of cells of nonrenal origin, such as Chinese hamster ovary cells (Kaneko et al., 1990), cultured human retinal pigment epithelial cells (Henry et al., 1993), and human embryonic epithelial cells (Ferraretto et al., 1993). Transient transfection studies with luciferase or chloramphenicol acetyltransferase reporter constructs, containing various 5'-flanking regions of aldose reductase gene, identified the osmotic response element mediating this hyperosmotic stress-induced increase in transcription of aldose reductase gene (Ferraris et al., 1996; Daoudal et al., 1997; Ko et al., 1997). Studies on the factors that interact with these response elements and augment the transcription of aldose reductase gene are now in progress and may provide insight into the regulatory mechanisms of the gene expression. Nevertheless, physiological implications of the osmoregulatory role for aldose reductase in nonrenal cells is still unknown.

### C. Unique Tissue Distribution Pattern of Aldose Reductase

Recent investigations disclosed the unexpected distribution pattern of aldose reductase not only in different species but in tissues other than "target" organs of diabetic complications. In mouse, aldose reductase mRNA

was most abundantly expressed in the testis, whereas a very low level of the transcript was detected in the sciatic nerve and lens (Gui et al., 1995). These results suggest that mouse aldose reductase may possess a significant role in the testicular metabolism. On the other hand, the low expression of the enzyme in the nerve and lens was in marked contrast with the findings in rat, which indicated the localization of the enzyme transcript in these "target" organs of diabetic complications (Nishimura et al., 1988). Consistent with these findings is the absence of cataract formation during the course of hyperglycemia in mouse (Varma and Kinoshita, 1974), in contrast with the finding in rat, the first experimental model of sugar cataract formation (Kinoshita and Nishimura, 1988). Immunoblot and immunohistochemical analyses in rat tissues further showed high levels of aldose reductase protein in the adrenal gland and various reproductive organs, including the granulosa cells of rat ovary (Iwata et al., 1990). Of particular interest is the fact that cyclic changes in the expression and localization of aldose reductase were observed in rat ovary during the estrous cycle (Iwata et al., 1996). These changes in the enzyme expression were indicated to be under hormonal control, and the study suggests another functional role of aldose reductase in the female reproductive organ, which can be deranged under diabetic conditions.

### D. Diverse Substrates for Aldose Reductase

Other lines of investigation have demonstrated that aldose reductase exhibits broad substrate specificity for both hydrophilic and hydrophobic aldehydes. Aldose reductase and the structurally related enzyme in the aldoketo reductase family, aldehyde reductase, both catalyze the reduction of biogenic aldehydes derived from the catabolism of the catecholamines and serotonin by the action of monoamine oxidase (Turner and Tipton, 1972; Tabakoff et al., 1973; Wermuth et al., 1982). These two enzymes also catalyze the reduction of isocorticosteroids, intermediates in the catabolism of the corticosteroid hormones (Wermuth and Monder, 1983). Recently, aldose reductase in the adrenal gland was reported to be a major reductase for isocaproaldehyde, a product of sidechain cleavage of cholesterol (Matsuura et al., 1996).

Apart from these findings, molecular cloning of bovine testicular  $20\alpha$ -hydroxysteroid dehydrogenase cDNA incidentally revealed that the deduced amino acid sequence of the enzyme is identical with bovine lens aldose reductase (Warren et al., 1993). The result implies that a biologically active progesterone as well as  $17\alpha$ -hydroxyprogesterone, a major precursor of the androgens, estrogens, and glucocorticoids, are endogenous substrates for bovine aldose reductase. To examine whether this finding is also applicable to mouse aldose reductase, we tested various steroids as substrates in the kinetic analysis of purified recombinant enzyme. Although progesterone and  $17\alpha$ -hydroxyprogesterone potently com-

peted with the substrate binding, the mouse enzyme did not show any steroid dehydrogenase activity (Gui et al., 1995). The lack of catalytic activity for the steroid substrate in mouse aldose reductase could be attributed to a subtle difference in the amino acid residues constituting the active site, whereas low inhibition constants for these steroids introduced the possibility that availability of glucose to mouse enzyme may be significantly affected in the tissues containing high levels of endogenous steroids. By contrast, human aldose reductase was recently reported to exhibit the reductase activity for  $17\alpha$ -hydroxyprogesterone with the similar kinetic parameters to bovine enzyme (Petrash et al., 1996). It can therefore be postulated that the functional or physiological roles of aldose reductase differ considerably among animal species as well as among tissues.

In a series of aldehyde substrates for human aldose reductase investigated, isocorticosteroids (Wermuth and Monder, 1983) and isocaproaldehyde (Matsuura et al., 1996), both with  $K_M$  values of approximately  $1 \mu\text{M}$  or less, are the best physiological substrates known to date. The next preferred substrates for aldose reductase may be aldehydes derived from biogenic amines (Turner and Tipton, 1972; Tabakoff et al., 1973) and methylglyoxal, a toxic aldehyde produced nonenzymatically from triose phosphate and enzymatically from acetone/acetol metabolism (Vander Jagt et al., 1992).  $17\alpha$ -hydroxyprogesterone (Petrash et al., 1996) and 4-hydroxynonenal (Vander Jagt et al., 1995), a reactive aldehyde produced by oxidative damage to unsaturated fatty acids, are also excellent substrates for the enzyme with  $K_M$  values of  $20\text{--}30 \mu\text{M}$ . Another line of study demonstrated that 3-deoxyglucosone, one of the crosslinking agents formed as intermediates in nonenzymatic glycation, is a good

substrate for aldose reductase (Feather et al., 1995). Aldose reductase also catalyzes the reduction of acrolein, a highly reactive and mutagenic molecule generated during lipid peroxidation and as a metabolic by-product of cyclophosphamide (Kolb et al., 1994). Both 3-deoxyglucosone and acrolein exhibited a similar range of  $K_M$  values ( $40\text{--}80 \mu\text{M}$ ) in the kinetic analysis (Vander Jagt et al., 1996). Whereas glucose is one of the endogenous substrates for aldose reductase, comparison with other endogenous aldehydes unequivocally indicates that glucose is a rather poor substrate with a  $K_M$  value of  $70 \text{ mM}$  (Vander Jagt et al., 1990). The interpretation of these findings is that aldose reductase in the adrenal gland and reproductive organs may normally participate in the synthesis and catabolism of steroid hormones, whereas it is involved in the metabolism of biogenic amines in the central nervous system. The enzyme may also act as extrahepatic detoxification enzyme in various tissues (fig. 3). Thus the significance of aldose reductase in the polyol pathway may be quite limited under nondiabetic conditions: it provides an osmolyte sorbitol in the renal medulla and supplies fructose as an energy source of sperm in the seminal vesicle.

Recent studies hence illustrate the diversity in biological significance of aldose reductase in different tissues and in different animal species. The interactions of the inhibitors with aldose reductase in various organs along with other structurally related proteins in aldo-keto reductase family, may become a potential source of their ineffectiveness and/or side effects when drugs are administered to diabetic patients for a prolonged period of time. Experimental data on the efficacy and side effects of inhibitors obtained from animal models should be cautiously interpreted, as significant species-specific dif-

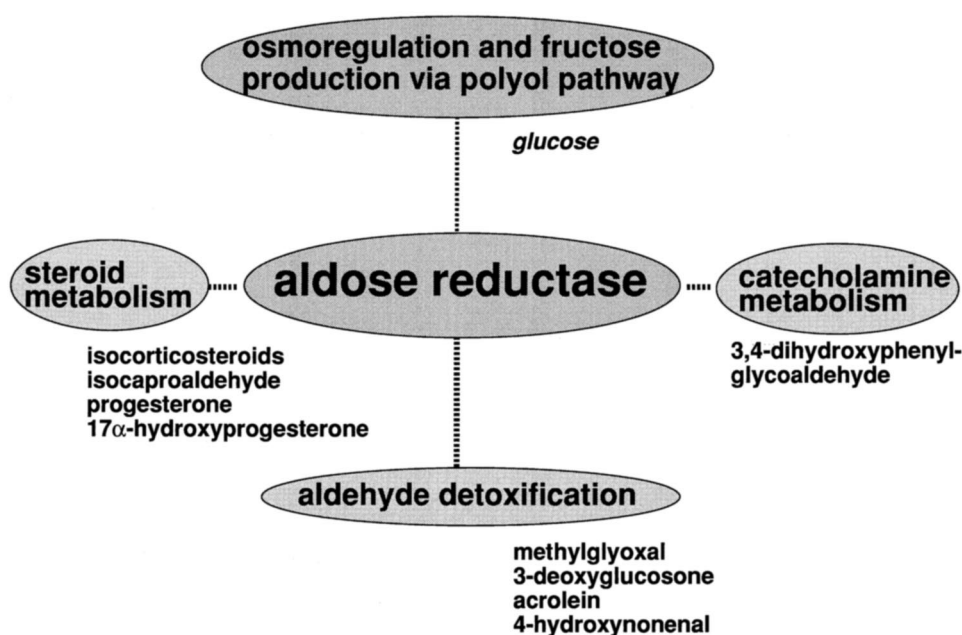


FIG. 3. A diversity of substrates and putative physiological roles of aldose reductase. See Section III for further detail.

ferences in the localization and in physiological functions of aldose reductase were noted. Nevertheless, it should be appended that aldose reductase is not the only enzyme participating in most of the above-mentioned pathways of endogenous aldehyde metabolism. The suppression of aldose reductase activity with enzyme inhibitors may thus have moderate effects on such aldehyde metabolism aside from polyol pathway.

#### IV. Aldose Reductase in Glucose Toxicity

##### A. Effect of Accelerated Polyol Pathway

For the past 4 decades, a wealth of experimental data has been accumulated on the role of accelerated polyol pathway in the process leading to the early tissue damage observed under hyperglycemia (Kinoshita and Nishimura, 1988). In the ocular lens, the accumulation of polyol induces hyperosmotic swelling and deranges the cell membrane, resulting in the leakage of amino acids, glutathione, and myoinositol to provoke cataract formation (Nagata et al., 1989). In other "target" organs of diabetic complications, however, such osmotic stress is currently considered to play a minor role in the tissue damage. Instead, depletion of cofactors used in the pathway by accelerated flux of glucose is postulated to elicit various metabolic disturbances in those tissues. Under normoglycemic conditions, polyol pathway accounts for approximately 3% of glucose utilization (Morrison et al., 1970), whereas more than 30% of glucose is metabolized through this pathway under hyperglycemia (González et al., 1984). The increased flux of glucose through this pathway and consequent expenditure of cofactors for aldose reductase (NADPH) and sorbitol dehydrogenase (NAD<sup>+</sup>) lead to a redox state change and a cascade of interrelated metabolic imbalances. Substantially affected are activities of glutathione reductase and nitric oxide (NO) synthase because of the depletion of the cofactor NADPH. As glutathione reductase is an antioxidative enzyme that maintains the level of tissue glutathione, the overall effect would be the increased susceptibility to oxidative stress under diabetic conditions. Indeed increased susceptibility to H<sub>2</sub>O<sub>2</sub> along with a reduced level of glutathione was reported in the endothelial cells cultured in high glucose medium (Kashiwagi et al., 1994). Similarly, the production of NO from L-arginine by NO synthase is suppressed resulting from the depletion of NADPH, thereby reducing the release of NO to elicit microvascular derangement and the slowing of nerve conduction (Cameron et al., 1993; Stevens et al., 1994).

In the retina of experimental diabetic animal models, the early lesion emerges in vascular component. Consistent with this observation, the localization of aldose reductase in retinal microvessels was demonstrated in various animal species including human trypsin-digested retina (Akagi et al., 1983; Kennedy et al., 1983; Hohman et al., 1989). Treatment with aldose reductase

inhibitors was shown to prevent capillary basement membrane thickening, the early structural lesion observed in the retina (Frank et al., 1983; Robison et al., 1983, 1989; Chakrabarti and Sima, 1989). By contrast, whether perturbation in the vasculature or metabolic disturbance in the neural cells contributes primarily to the development of diabetic neuropathy has long been controversial. In 1959, Fagerberg first described the vascular involvement in the process leading to the structural lesion observed in diabetic neuropathy (Fagerberg, 1959). Later, however, most investigators in the field turned their attention to metabolic disturbances and ensuing neurochemical alterations in diabetic nerve. Effects of hyperglycemia on the polyol pathway activity and associated neurochemical derangement have been extensively studied in the peripheral nerve of experimental animals (Tomlinson et al., 1984; Greene et al., 1987; Nishimura et al., 1987). In fact, aldose reductase immunoreactivity was found in the paranodal cytoplasm of Schwann cells as well as in pericytes and endothelial cells of endoneurial capillaries (Chakrabarti et al., 1987).

##### B. Transgenic Animal Model

To investigate the effect of enhanced polyol pathway activity on the process leading to early tissue damage in diabetes, we made a transgenic animal model expressing human aldose reductase (Yamaoka et al., 1995; Yagihashi et al., 1996). As the transgene was driven by the murine major histocompatibility complex class I antigen, H-2K<sup>d</sup> gene promoter, a broad expression of human aldose reductase was demonstrated in most of the mouse tissues examined. In the sciatic nerve, immunohistochemical analysis using antibody, specific for human enzyme, indicated that aldose reductase was localized in Schwann cells, axons, and endothelial cells of endoneurial microvessels in the transgenic mice (Yagihashi, 1995). When these mice were fed with the diet containing 30% galactose, a significant level of galactitol was accumulated in the nerve. In this experiment, galactose feeding was substituted for induction of hyperglycemia so as to evaluate the direct effect of aldose reductase activity overexpressed in transgenic mice. This is because galactose is a better substrate for aldose reductase and galactitol is not further metabolized by sorbitol dehydrogenase (Hayman and Kinoshita, 1965). The level of galactitol in nerves of galactose-fed transgenic mice was 10 times higher than that in galactose-fed nontransgenic littermate mice, indicating the functional expression of the transgene integrated in the nerve tissue. The biochemical derangement in galactose-fed transgenic mice was accompanied by a significant delay in motor nerve conduction velocity, as well as significant atrophy in the myelinated fiber as was determined by morphometric analysis. Our transgenic mouse work, therefore, suggests the primary role of the augmented aldose reductase activity in the development of functional and

structural abnormalities in the peripheral nerve of the diabetic animal model.

### C. Hemodynamic Abnormalities in Diabetic Neuropathy

On the other hand, renewed attention has recently been paid to the vascular involvement in the pathogenesis of diabetic neuropathy. Systemic investigation of the distribution of myelinated fiber loss from proximal to distal levels of the lower limb nerves in diabetic patients indicated the involvement of ischemia in the process leading to these morphological changes (Dyck et al., 1986). In fact, endoneurial hypoxia resulting from reduced nerve perfusion was demonstrated in diabetic rat (Tuck et al., 1984) and later in diabetic patients with neuropathy (Newrick et al., 1986). Of particular interest is the fact that administration of an aldose reductase inhibitor significantly improved the blood flow in the nerve tissue measured by laser-Doppler flowmetry, and a strong correlation between inhibitor-mediated improvement in the blood flow and in nerve conduction velocity was observed (Cameron et al., 1994). The possible link between metabolic disturbances elicited by hyperglycemia and such hemodynamic abnormalities in the nerve tissue of diabetic animals is the accelerated polyol pathway flux in the vascular cells. Enhanced polyol pathway activity would provoke impaired production of NO and other bioactive molecules in vascular endothelial cells. Experimental data also indicate that the deficit in NO release was prevented by aldose reductase inhibitor in the aorta of diabetic rats (Cameron and Cotter, 1992).

Intriguingly, however, there emerged unexpected data on the dose-response relationships for aldose reductase inhibition and various experimental findings in these animal experiments. Poor agreement was demonstrated between functional deficits and biochemical changes in the nerve of diabetic rats (Cameron et al., 1994). Compared with the dosage of aldose reductase inhibitor to correct the biochemical changes in the nerve, nearly 10 times higher dosage was necessary to correct the blood flow and conduction velocity of diabetic rat nerves. Thus, nerve conduction velocity is much less sensitive to the inhibitor treatment, and the improvement in nerve conduction does not correlate with the improvement in polyol pathway metabolites in the nerve. In this context, it should be noted that the levels of polyol pathway metabolites reflect the mass derived from axons and Schwann cells, the dominant components in the peripheral nerve. The mechanisms underlying this significant disparity in the inhibitor effects are still unknown. In any case, such difference in the sensitivity may at least partly explain the modest effect of aldose reductase inhibitors on nerve conduction velocity deficits reported in clinical trials. The involvement of aldose reductase in diabetic vascular abnormalities has been a matter of recent attention, and much remains to

be clarified on the discrepancy in the dose-response relationship of inhibitors between neural and vascular components of the peripheral nerve. It should be noted, however, that the above provocative findings on dose-response relationships of aldose reductase inhibitors for corrections of the blood flow and nerve conduction velocity have not been verified by other laboratories.

### D. Aldose Reductase and Other Factors in Glucose Toxicity

Along with the increased flux of glucose through the polyol pathway, there are other putative mechanisms that may take part in the toxic effects of hyperglycemia (fig. 4). Among the well-documented factors are activation of protein kinase C (Lee et al., 1989; Williams, 1995), enhanced nonenzymatic glycation (Brownlee et al., 1984), and augmentation of oxidative stress (Sato et al., 1979; Hunt et al., 1993). Some of these are postulated to be correlated with each other. Activation of protein kinase C was reported in vascular smooth muscle and endothelial cells after the exposure to hyperglycemia. Increased incorporation of [<sup>14</sup>C]glucose into diacylglycerol (DAG) indicated that the enzyme activation was elicited by increased de novo synthesis of DAG through glycolytic pathway (Craven et al., 1990). The rise in NADH/NAD<sup>+</sup> ratio via enhanced polyol pathway may also facilitate the DAG synthesis by increasing the availability of dihydroxyacetone phosphate as well as favoring its reduction to *sn*-glycerol 3-phosphate, the intermediates of DAG synthesis (Pugliese et al., 1991). The increased protein kinase C activity would attenuate contractile responses of aortic vascular smooth muscle cells to such pressor hormones as angiotensin II and arginine vasopressin. The activation of protein kinase C increases sodium-proton antiport activity that regulates intracellular pH, cell growth, and differentiation and also augments expression of various matrix proteins such as fibronectin, type IV collagen, and laminin (Williams, 1995). All these biochemical changes could be relevant to diabetes-induced vascular dysfunction. This phenomenon was demonstrated in several tissues including retina, aorta, and renal glomeruli. Recently a specific inhibitor for the  $\beta$  isoform of protein kinase C was shown to ameliorate vascular dysfunctions in diabetic rats (Ishii et al., 1996). In peripheral nerve, however, the DAG level was reduced in diabetic rats (Zhu and Eichberg, 1993; Ido et al., 1994) and an activation of protein kinase C has not been reported in diabetic nerves. The underlying mechanisms of this tissue specific activation of protein kinase C have to be further elucidated. Although the new protein kinase C inhibitor can be another candidate drug for therapeutic usage, such difference in the activation pattern among the "target" organs and localization of the enzyme  $\beta$  isoform in tissues other than "target" organs of diabetic complications may hinder its general application to diabetic individuals.

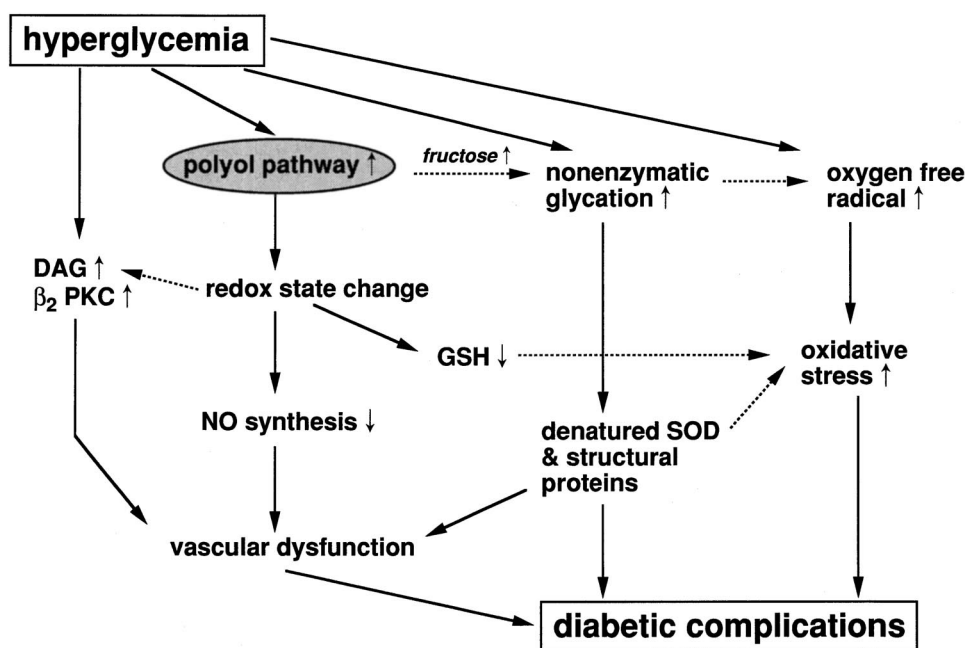


FIG. 4. A schematic diagram of possible interactions among factors involved in the pathogenesis of diabetic complications. (DAG), diacylglycerol; ( $\beta_2$  PKC),  $\beta_2$  isoform of protein kinase C; (NO), nitric oxide; (GSH), glutathione; (SOD), superoxide dismutase.

Under hyperglycemic conditions, nonenzymatic glycation of structural proteins is enhanced, and advanced glycation end-products accumulate in diabetic tissues. As a glycation agent, fructose is more potent than glucose (Stevens et al., 1977), and the formation of fructose is augmented because of the accelerated flux of glucose through the polyol pathway. Increased nonenzymatic glycation has been shown to alter the structure and function of various macromolecules in the tissue, causing basement membrane thickening, demyelination, and impaired axonal transport as a result of the glycation of myelin, tubulin, and neurofilaments (Brownlee et al., 1988). Longterm treatment with aminoguanidine, an inhibitor of the glycation process, was effective in retaining the functional and structural integrity in the vascular and peripheral nerve tissues in diabetic rats (Hammes et al., 1991; Yagihashi et al., 1992). However, interpretation of these data is not straightforward, as aminoguanidine was later shown to inhibit the NO synthase (Corbett et al., 1992).

Various mechanisms are postulated to account for augmented oxidative stress in diabetes. A generation of oxygen free radicals was enhanced because of auto-oxidation of glucose. The protection against oxidative stress was attenuated because of reduced glutathione availability and inactivation of superoxide dismutase. Vascular dysfunction and resulting derangement in tissue perfusion under diabetic conditions would induce ischemia and reperfusion process, which further generate oxygen free radicals (McCord, 1985). On the other hand, it has been generally accepted that advanced glycation participates in the production of oxygen free radicals (Hunt et al., 1993). Inactivation of superoxide dismutase in dia-

betes was demonstrated to result from glycation of the two lysine residues on the enzyme protein (Arai et al., 1987). The polyol pathway may act upon this enhanced glycation process, supplying a reactive glycation agent fructose. Reduced glutathione availability under hyperglycemia is attributed to the accelerated polyol pathway flux, depleting the cofactor NADPH for glutathione reductase (fig. 1).

In this context, most of the putative mechanisms implicated in the toxic effects of hyperglycemia can be interrelated to each other and linked to enhanced polyol pathway activity. The crucial question yet left unanswered is as to what extent the polyol pathway participates in such an interrelated process leading to diabetic complications.

## V. A Potential Target for the Prevention of Diabetic Complications

### A. Clinical Trials of Aldose Reductase Inhibitors

A promising effect of aldose reductase inhibitor on nerve conduction velocity was reported more than a decade ago. When diabetic patients without any symptomatic neuropathy were treated with the aldose reductase inhibitor sorbinil, significant improvement in the conduction velocity was observed in all three nerves tested: the peroneal motor nerve, the median motor nerve, and the median sensory nerve (Judzewitsch et al., 1983). Subsequently, numerous clinical studies were carried out to evaluate the efficacy of sorbinil. However, the overall effect turned out to be disappointingly modest, possibly because of the difference in the study design, subjects with various degrees of symptomatic neu-



ropathy, and neurophysiological parameters examined as study endpoints (Fagius et al., 1985; O'Hare et al., 1988; Sima et al., 1988). The major adverse reaction of sorbinil was a hypersensitivity reaction in the early weeks of therapy, which is similar to that seen with other hydantoin. Although no clinically important adverse reaction was observed with ponalrestat, the succeeding aldose reductase inhibitor of a different chemical structure, its beneficial effect failed to be proved in randomized controlled study (Krentz et al., 1992). After the clinical trials, however, it was shown that ponalrestat did not penetrate the human nerve at doses sufficient to decrease the nerve sorbitol levels (Greene and Sima, 1993). The efficacy of another class of inhibitor, tolrestat, was modest in diabetic patients already symptomatic of neuropathy (Boulton et al., 1990), although the progress of mild diabetic autonomic and peripheral neuropathy could be halted (Giugliano et al., 1993, 1995). The only adverse reaction reported on tolrestat was an increase in serum levels of alanine aminotransferase or aspartate aminotransferase, although some patients in the placebo group also exhibited similar clinical findings during the study (Nicolucci et al., 1996). Because of the inability to demonstrate efficacy on the nerve conduction velocity in the multicenter double-blind studies on diabetic neuropathy, however, the clinical development of tolrestat was eventually withdrawn.

### *B. Variable Levels of Aldose Reductase in Diabetic Patients*

Substantial variations in the levels of aldose reductase expression in various tissues exist among individuals with or without diabetes. Marked variability in aldose reductase activity was reported for enzyme preparations isolated from human placentas (Vander Jagt et al., 1990). Aldose reductase purified from erythrocytes exhibited a nearly three-fold variation in activity among diabetic patients (Hamada et al., 1991). Such differences in the activity of aldose reductase may influence the susceptibility of patients to glucose toxicity via acceleration of polyol pathway when these individuals are maintained under equivalent glycemic control. To test this hypothesis, it is necessary to determine the levels of aldose reductase in numerous diabetic subjects. In the previous studies, investigators examined variations in aldose reductase by isolating the enzyme from placenta or erythrocytes and assaying its activity (Vander Jagt et al., 1990; Hamada et al., 1991). The isolation of the enzyme was necessary because of the presence of other structurally related members of aldo-keto reductase family, particularly aldehyde reductase, in crude tissue preparations. These enzymes share overlapping substrate specificity with aldose reductase. Determination of the enzyme activity in human subjects was therefore quite laborious. Moreover, the tedious isolation procedures have problems of possible variations in enzyme recovery when the activity is to be compared among a

group of individuals. By contrast, our newly developed immunoassay method using a specific antibody against aldose reductase could circumvent such difficulties (Nishimura et al., 1993). The amount of the enzyme determined by the immunoassay highly correlates with the activity of aldose reductase isolated from the erythrocytes of the same individuals (Nishimura et al., 1994a).

By using this assay method, we investigated the association between the aldose reductase level in the erythrocyte, and various clinical parameters determined in patients with non-insulin-dependent diabetes mellitus (NIDDM). Several-fold difference in the erythrocyte enzyme level was depicted among diabetic patients, whereas no significant difference in the mean enzyme level was demonstrated between the healthy and diabetic individuals. The enzyme level did not correlate with age, duration of diabetes, fasting blood glucose, or glycosylated hemoglobin (HbA<sub>1c</sub>) levels, which represent glycemic control of the patient. However, data obtained from two different groups of diabetic subjects suggest that a high level of erythrocyte aldose reductase may affect the susceptibility and prognosis of diabetic retinopathy (Nishimura et al., 1994b, 1997). In another study group, 95 NIDDM patients were classified according to the results of seven nerve function tests, and the association between the enzyme level and the clinical findings was investigated (Ito et al., 1997). The erythrocyte aldose reductase level was significantly higher in those patients showing overt neuropathy compared with those without demonstrable neuropathy (fig. 5). Multivariate logistic regression analysis identified that a higher level of aldose reductase is one of the independent risk factors for overt neuropathy. Accordingly, these results support our earlier hypothesis that a difference in the level of aldose reductase is responsible for the susceptibility of diabetic patients to toxic effects of glucose. Along with our findings, the activity of aldose reductase fractionated from the erythrocytes was reported to be significantly higher in IDDM patients with complications compared with those showing no sign of complications (Hamada et al., 1993). Recently, increased levels of aldose reductase protein were also demonstrated by immunoblot analysis in the mononuclear cells isolated from IDDM patients with apparent diabetic complications (Ratliff et al., 1996).

The question as to what is responsible for high or low expression of aldose reductase in human tissue is not only of scientific, but also of great clinical significance when targeting the enzyme for therapeutic intervention of diabetic complications. The level of aldose reductase expressed in the erythrocyte seems to be stable, as no apparent alteration in the enzyme level was observed during the follow-up period of 12 months in the studied patients (Ito et al., 1997). In this study, the enzyme level remained unchanged irrespective of improved or stably high HbA<sub>1c</sub> levels during the follow-up period. These

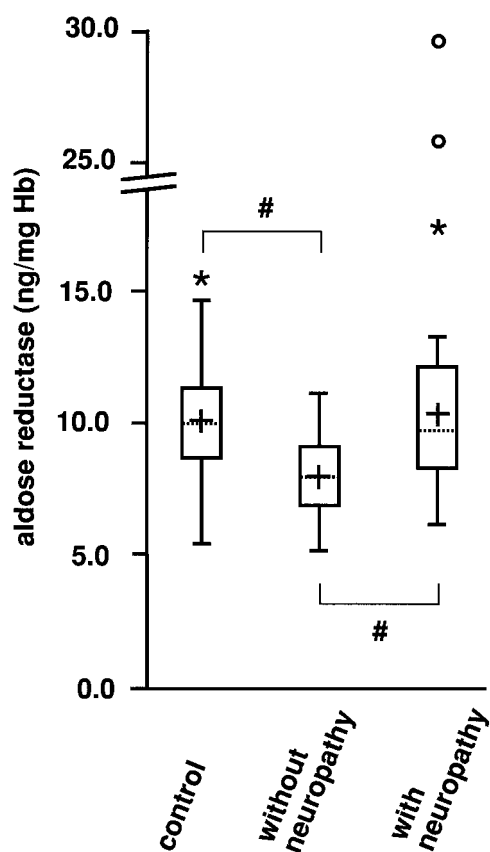


FIG. 5. Box-plots of erythrocyte aldose reductase levels in 76 healthy individuals (control) and 95 non-insulin-dependent diabetes mellitus patients determined by immunoassay. Based on the results of nerve function tests, patients were classified into those without demonstrable neuropathy (without neuropathy;  $n = 45$ ) and those with overt neuropathy (with neuropathy;  $n = 50$ ). The mean enzyme levels expressed per mg of hemoglobin (Hb) are represented by + in the plots. #  $p < 0.01$ , by Bonferroni  $t$ -tests, whether the 3 extreme values in "with neuropathy" group were excluded or included. No statistical difference was demonstrated between the healthy controls and the total diabetic patients. Modified from Ito T, Nishimura C, Takahashi Y, Saito T, and Omori Y (1997) The level of erythrocyte aldose reductase: a risk factor for diabetic neuropathy? *Diabetes Res Clin Pract* 36:161–167. With permission from Elsevier Science Ireland Ltd, Bay 15K, Shannon Industrial Estate, Co. Clare, Ireland.

findings indicate that the expression of the erythrocyte enzyme is unaffected by the glycemic control of the patients. It can, therefore, be speculated that different levels of aldose reductase observed in diabetic patients may be genetically determined. To explore this possibility, we examined two regions on the aldose reductase gene relevant to the enzyme expression: the promoter region containing a TATA box (Wang et al., 1993), and the region containing the recently identified osmotic response sequences (Ko et al., 1997). However, in the DNA sampled from 700 NIDDM patients with different enzyme levels in the erythrocyte, we found no change in either of these regions associated with differences in the expression of aldose reductase levels (Nishimura, unpublished observations). Thus the reason for the variable expression of aldose reductase in human subjects has yet to be elucidated. The understanding of the mechanisms defining the expression levels in the targeted

tissues may lead to new avenues of preventive therapy for diabetic complications.

A hypothesis as to whether the high enzyme level predisposes the patients to the development of complications has to be further tested by the prospective study carried out through the prolonged time course of diabetes. Also to be considered is the relevance of the aldose reductase level in the erythrocyte in predicting the enzyme level in the "target" tissues of diabetic complications. Whether a high level of enzyme expression in the erythrocyte reflects the level in the different cell lineage has to be determined. It will take some time before all the data become available; nonetheless, a high level of aldose reductase in the erythrocyte was demonstrated to be a risk factor for vascular and neural derangement observed in diabetic patients. Identification of a subset of patients who have a high level of aldose reductase expression, and thereby are more susceptible to toxic effects of glucose, may enable us to target these patients for clinical intervention trial by use of new aldose reductase inhibitors. The data on the enzyme levels may also aid in the optimization of administration of the inhibitors to match the extent of enzyme suppression when exploring their efficacy in diabetic individuals.

## VI. Conclusions

Recent progress in the understanding of biochemical correlates of aldose reductase has paved the way for designing and screening specific inhibitors of this enzyme that can be used as therapeutic agents for diabetic complications. The tertiary structure of aldose reductase, including the active site and the interaction with inhibitors of diverse chemical structures has been resolved. Abundant amounts of purified human enzyme are now available by recombinant technique. Nevertheless, much still remains to be elucidated regarding the pathophysiological significance of the enzyme and the regulatory mechanisms of aldose reductase expression in various human tissues.

In diabetic animal models, promising effects of aldose reductase inhibitors were demonstrated. Most of the clinical trials carried out so far, however, produced rather modest or disappointing effects of the inhibitors on the functional and morphological improvements in diabetic neuropathy. There could be several reasons that account for the disparity in the inhibitor effects observed between animal and clinical studies. Possible explanations include the chronic nature of diabetes in human subjects and the ensuing loss of ability to reconstitute the structural derangement once triggered under hyperglycemia. High variability in the neurological measurements as endpoints for the inhibitor effects should be considered as well. Yet there may be other reasons for disappointing results observed with clinical trials. For example, other structurally related members of aldose reductase family, coexisting in the "target" tissues, may have interfered with the action of inhibitors,

quenching their action against aldose reductase. We still do not know the relative abundance of the aldo-keto reductase family, such as aldehyde reductase. These enzymes are colocalized in human tissues and the latter may interfere with the action of inhibitors to suppress aldose reductase.

Moreover, inappropriate dosage of inhibitors may have been used resulting in a failure to observe improvements in the endpoint of these clinical studies, as the dosage to correct the nerve conduction velocity may be significantly higher than that to substantially reduce polyol pathway metabolites in neuronal tissues. Whether nearly 100% inhibition of the polyol pathway metabolites is required for an improvement of nerve conduction velocity or if there are hitherto unknown reasons needs to be elucidated. Species difference in the structure and distribution of the enzyme and ensuing differences in sensitivity to inhibitors may also contribute to the disappointing clinical results obtained with inhibitors that have marked effects in animal studies. Lastly, the efficacy of aldose reductase inhibitors may depend on the enzyme level expressed in diabetic individuals. The variable levels of the enzyme expressed in the "target" tissues may affect the extent of involvement of the polyol pathway in the pathogenetic mechanisms of diabetic complications. If multiple mechanisms are involved in the pathogenesis of diabetic complications, the extent of the effectiveness of aldose reductase inhibitors is likely to be determined by the extent of the polyol pathway involvement in the toxic effects of hyperglycemia. This extent is likely to be variable among individuals having different levels of aldose reductase in "target" tissues.

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