Aldose Reductase in The Retina

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Abstract: Aldose reductase (E.C. 1.1.1.21), an intracellular enzyme of polyol pathway, catalyzes NADPH-dependent reduction of glucose to sorbitol. Under normoglycemia, most of the cellular glucose is phosphorylated into glucose-6-phosphate by hexokinase. A minor part of non-phosphorylated glucose enters the polyol pathway, the alternate route of glucose metabolism. However, under hyperglycemia, because of saturation of hexokinase with ambient glucose, aldose reductase is activated, leading to excessive production of sorbitol. Intracellular accumulation of sorbitol is thought to result in irreversible damage. In the diabetic eye, the increased sorbitol accumulation in retina has been implicated in the pathogenesis of retinopathy, characterized by pericyte loss, basal membrane thickening, the major ocular complications of diabetes. Nearly all diabetic subjects have the same degree of retinopathy after 20 years of diabetes. 50% of patients with insulin dependent diabetes mellitus have proliferative retinopathy after 15 years. In addition, macular edema frequently produces central vision loss and blindness most commonly in non-insulin dependent diabetes mellitus. Therefore, aldose reductase enzyme inhibition is becoming one of the therapeutic strategies that have been proposed to prevent or ameliorate long-term diabetic complications.

Keywords: Aldose reductase, glucose, retina hyperglycemia, blindness.

INTRODUCTION

Aldose reductase (AR) is one of the most thoroughly studied of aldo-keto reductase due to its putative involvement in the pathogenesis of diabetic eye diseases [1]. AR catalyzes NADPH–dependent reduction of glucose to sorbitol (polyol pathway), the first step of sorbitol pathway (Fig. 1). A large body of evidence, derived principally from experimental animal studies, supported the hypothesis that enhanced metabolism of glucose through polyol pathway results in biochemical imbalance associated with diabetic complications [2]. Chronic hyperglycemia leads to many changes in target tissues of diabetic patients including accumulation of excess polyol, alteration in the relative abundance of oxidized and reduced nicotinamide co-enzyme, change in the levels of glycolytic intermediates and loss of glutathione [3].

It is well established that high blood glucose levels greatly influence the development and progression of diabetic retinopathy [4]. Most cells of the retina are affected by the metabolic abnormalities of diabetes [5] but the sight–threatening manifestations of diabetic retinopathy are ultimately attributable to capillary damage [6]. Abnormal permeability of barrier capillaries can cause macular edema and capillary closure leading to ischemia and unregulated angiogenesis [7]. Retinopathy is the most severe complication of diabetes mellitus because it can result in irreversible blindness [8]. Therefore, elucidating the

Fig. (1). The polyol pathway; glucose-6-phosphate [2].

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mechanism for initiating diabetic retinopathy is important for prevention or treatment.

Aldose reductase inhibition represents an attractive strategy for the prevention of diabetic complications. The beneficial effect of aldose reductase inhibition in preventing or substantially delaying the onset of diabetic complications in experimental models provides strong support to this hypothesis [2]. Moreover, transgenic animal studies have shown that over expression of aldose reductase confers an increased susceptibility to diabetic cataract [9] and morphological changes in retinal vasculature similar to those observed in human diabetic retinopathy [10].

A. Retina

The retina is composed of the tissues arising from the optic vesicle and consists of a pigment epithelium layer, which is derived from the outer layer of the optic cup and a complex sensory layer, which is derived from the inner layer [11].

1. Retinal Pigment Epithelium

The retinal pigment epithelium (RPE) is a single layer of hexagonal, deeply pigmented cells with a regular arrangement that forms a sheet between Bruch's membrane and the photoreceptor layer of the sensory retina. This epithelium extends from the margin of the optic disc to the ora serrata and then it continues with the pigment epithelium of the ciliary body [12].

In the posterior part of the eye, the RPE cells are fairly uniform in size and shape and measure approximately 16 µm in diameter. In the mid periphery and equatorial areas, the cells are thinner and larger in diameter. In the far periphery, the cells are variable in size and this variability increases with age [13].

The cytoplasm of the RPE cells contains oval or rounded pigment granules that are located predominantly near the apical side. Other pigment granules, composed of lipofuscin, are found in the cytoplasm and accumulate in the cells during the process of aging, particularly in the macular area [14].

The functions of the RPE are mainly the storage of vitamin A and its conversion to a form that can be utilized by the photoreceptors for synthesis of rhodopsin and also phagocytosis and degradation of shed lamellar discs of the photoreceptors [15].

B. Aldose Reductase

Aldose reductase (AR) is a cytosolic enzyme present in most of the mammalian cells. The polyol pathway was first identified in the seminal vesicle by Hers [17], who demonstrated the conversion of blood glucose into fructose, an energy source for sperm cells. Later, the presence of sorbitol in diabetic rat lens was reported by Van Heyningen [18]. This work provided the basis for new research concerning the pathological role of AR and the polyol pathway in the development of diabetic complications. Several studies have suggested that increased reduction of glucose by AR contributes to the development of secondary diabetic complications [19]. During hyperglycemic event, the elevated glucose level enhances the activity of AR by increasing glucose flux through this pathway [20]. In fact, AR messenger ribonucleic acid (mRNA) is highly expressed in the rat lens, retina and sciatic nerve, the major target organs of diabetic complication [21]. The increased activity of AR results in decreased NADPH/NADP+ ratio, which has impact an on other NADPH-dependent enzymes, such as nitric oxide (NO) synthase and glutathione reductase [22]. The retarded activity of the antioxidant enzyme, glutathione reductase causes oxidative stress under diabetic conditions. Increased sorbitol flux through the polyol pathway causes increases in NADH/NAD+ ratio, which blocks the glycolytic pathway at the triose phosphate levels. Consequently, protein KinaseC (PKC) is activated and the formation of advanced glycation end products is increased. Advanced glycation end products are toxic. they cause pathological changes by disrupting protein function and interfering with cellular receptors and have been implicated in the etiology of diabetic complications [23].
Molecular cloning techniques have identified many amino acid sequences for cellular proteins (39 proteins) as members of the aldo-keto reductase superfamily [34]. A wide variety of proteins from various species constitute this family, including aldehyde and xylene reductases from plants, yeast, and bacteria [35]. Nonetheless, the majority of this family is represented by mammalian aldehyde reductases, aldose reductases and hydroxysteroid dehydrogenases. These enzymes are now categorized into functionally and evolutionarily related groups based on their genetic origins [36, 37]. Thus, the superfamily of AKRs is organized into a series of families and subfamilies. While the overall structural features of the AKR family members are well conserved, subtle differences near the C-terminal domain are thought to be responsible for the difference in the substrate specificity among closely related enzymes.

2. Tertiary Structure of AR

X-ray crystallography and site directed mutagenesis studies have provided important insights into the structures of AR. Wilson et al. [38] showed the structure of human placenta AR. The enzyme molecule contains α/β barrels, which are still the most prevalent motif with a core of eight parallel β strands connected by peripheral α helices with a large hydrophobic active site (Fig. 2). The structure of active site of AR differs significantly from that of aldehyde reductase [39]. However, it has been shown that the coenzyme binding site is the same for AR and aldehyde reductase [2]. The NADP⁺ co-enzyme molecule is bound to the carboxyl terminus of the β barrel in an extended conformation. The nicotinamide ring is centered in the active site cavity. This highly hydrophobic active site pocket is formed by aromatic residues (Trp 20, Tyr 48, Trp 79, Trp 111, Phe 121, Phe 122, and Trp 219) apolar residues (Val 47, Pro 218, Leu 300 and Leu 301) and polar residues (Glu 49, Cys 298 and His 110). The pyrophosphate bridge of NADPH is tied down by residues 214-230 amino acids, which hold NADPH tightly in place [40]. The interactions that favor the binding of NADPH over NADH are due to Lys 263 and Arg 269 salt linked to the 2'-phosphate of adenosine moiety of NADPH [2].

3. The Catalytic Mechanism of AR

The proposed catalytic mechanism of AR consists of stereospecific transfer of the 4-pro-R hydrogen from the exposed C-4 of the nicotinamide of the bound NADPH to the carbon of the carbonyl group of the substrate, followed by the protonation of the substrate carbonyl oxygen by a proton donor group. Three residues within suitable distance of the C-4 were identified as the potential proton donor, including Tyr-48, His-110 and Cys-298. However, site directed mutagenesis studies implicated Tyr-48 as proton donor and that His 110 is involved in substrate recognition [41]. A hydrogen bonding interaction between the phenolic hydroxyl group of Tyr-48 and the ammonium side chain of Lys-77 is thought to help to facilitate hydrogen transfer [38]. Cachau et al. [42] further explained a new reaction path in which proton source was Tyr-48 and His-110 acted as a...
proton shuttle. After donation of a proton from Tyr-48, His-110\textsuperscript{+} intermediates formed, which resulted in the release of the proton to the substrate along with a hydride transfer from C-4 of nicotinamide of NADPH. A water bridge and correlation of movements between His-110 and Lys-66 oriented lysine toward polarized Tyr-48, which explained the loss of catalytic activity in Lys-77 mutants. The importance of Asp-43 was evident, as a salt bridge replaced the initial interaction of Lys with NADP\textsuperscript{+} after the reaction [42]. The reaction catalyzed by AR proceeds through an order sequential kinetic mechanism with NADPH binding first and NADP\textsuperscript{+} being released last [43]. The Asp 45-Lys 80-Tyr 50 system maintains an overall neutral charge on the enzymes active site between pH 6-8, allowing a proton to be transferred to the carbonyl oxygen of the substrate and a negative charge to develop on the tyrosyl hydroxide.

Elegant kinetic study showed that NADP\textsuperscript{+} release represents the rate-limiting step in the reaction of AR [44]. As the rate of co-enzyme release limits the catalytic rate, it can be seen that perturbations of the interactions that stabilize co-enzyme binding can have dramatic effects on V\textsubscript{max} [45].

4. Post-translational Regulation of AR Activity

Several investigators have reported evidence for an activated form of AR distinguishable from the native enzyme form by differences in \textit{Km} for aldehyde substrates and marked reduction to AR inhibitors [46]. Previous studies have shown that levels of activated form were found to be elevated in diabetic tissues. AR isolated from diabetic or hyperglycemic tissues is less susceptible to inhibition and is kinetically different from the enzyme purified from normal or euglycemic human or animal tissues [47]. These changes in the inhibitor sensitivity and the kinetic properties have been reported upon thiol oxidation of purified protein \textit{in vitro}, suggesting that diabetic changes in AR may be due to redox modification of its cysteine residues [48]. Due to the presence of a hyperreactive cysteine residue (Cys-298) located at the active site of the enzyme; AR is very sensitive to oxidants [49]. These functional changes are largely prevented if the enzyme is complexed with NADP(H) prior to the treatment with modifying agents. Since cysteine side chain is involved in the stabilization of the co-enzyme in such a way that the reactive thiol group becomes inaccessible. Therefore, the enzyme should be susceptible to modification through Cys-298 only during a catalytic cycle when nucleotide exchange occurs. Chandra et al. [50] showed that exposure to nitric oxide (NO) donor results in rapid and selective modification of AR at cysteine residue (Cys-298). These observations suggested that NO may be a physiological regulator of AR activity \textit{in vivo} and that changes in NO availability during diabetes could alter AR catalysis and consequently, the polyol pathway activity. Chandra et al. [51] showed that NO donors inhibit AR activity and sorbitol accumulation in erythrocytes. These observations are consistent with the view that NO is a physiological regulator of AR. The inhibitory effect of NO on AR catalysis and the polyol pathway activity was also evident in diabetes. The erythrocytes of diabetic animals when incubated with glucose or tissues isolated from diabetic rats accumulated significantly less sorbitol when treated with NO donors [51]. This observation provides strong evidence supporting a post-translational mechanism. However, the extent of inhibition of sorbitol formation in diabetic erythrocytes was much less than that observed in normal euglycemic cells, indicating that diabetes increased resistance of AR to the inhibitors. During diabetes, NO availability is decreased and therefore the net effect is the stimulation of sorbitol synthesis. However, it has been reported that NO could also affect the transcription of AR gene. Stimulation of vascular smooth muscle cells in culture with NO donors results in an increase in AR mRNA and inhibiting NO synthesis prevents the increase in AR [52]. Chandra et al. [51] suggested that transcriptional activation of AR gene \textit{in vivo} by NO is marginal and that this effect is overcome by the post-translational inhibition of AR activity. The AR gene is stimulated by the toxicity of high glucose [49] as well as growth factors, oxidants, mitogens [53] and cytokines [54]. Most of these stimuli are affected by diabetes and could change the AR expression during diabetes and therefore the stimulation of sorbitol synthesis. These observations explained the high level of AR in diabetic tissues.

5. General Kinetic Features of AR

Aldose reductase has been isolated and studied from a variety of species and tissues. Placenta and muscle are typical human tissue sources for enzyme purification [31, 55], although enzyme levels may be highest in the adrenal gland [56]. Kinetic and structural properties appear to be consistent, regardless of tissue source. In relative terms, glucose is a poor substrate for AR. The apparent \textit{Km} value for glucose has been reported by various groups to be in the range of 50-200 mM, well above normal physiological levels [55]. The catalytic efficiency of AR is approximately four orders of magnitude lower for D-glucose than for lipid-derived aldehydes, including 4-hydroxy-2-nonenal [57]. The poor apparent kinetic efficiency of AR with glucose suggests that the enzyme is maximally active only when intracellular hexose concentrations rise to abnormally high levels. Major endogenous substrates linked to metabolic diseases are glucose and galactose, which are converted to sorbitol and galactitol, respectively. Reactive sugars and trioses derived from aldohexoses, including methylglyoxal, glycosone and deoxyglucosone are excellent substrates for AR [57]. In diabetic animals, sorbitol production correlates with tissue levels of AR. Low levels of sorbitol accumulate in the diabetic mouse lens, which contains low levels of AR [58]. Rat and gerbil lenses, which are endowed with an abundance of AR, accumulate pathological levels of polyols when exposed to higher sugar levels [59].

C. Physiological Significance of AR

Current evidence suggests that AR contributes to metabolic imbalances associated with diabetes and its complications in the eye and peripheral nerves system [60]. While it is generally accepted that AR-mediated pathogenesis is dependent on chronically elevated ambient hexose levels as in diabetes mellitus and galactosemia. However, the beneficial role(s) of AR in the cells when hexose levels are normal are still under investigations [61]. AR gene expression is widespread, as evidenced by the presence of gene transcripts in a large number of human
tissues. It is suggested that the enzyme might function physiologically as a general housekeeping enzyme under normal conditions. In addition, new evidence points to a potential role for AR in cytokine-mediating signaling processes.

1. Osmoregulatory Role

The kidney is one of the richest tissue sources of AR, with most of the enzyme localized in the medullary portion [62] from which quantities of the enzyme are isolated for biochemical studies [63]. Sorbitol is one of the organic osmolytes that balance the osmotic pressure of extracellular NaCl, fluctuating in accord once with urine osmolality [64]. These findings therefore suggest the osmoregulatory role of AR in the renal homeostasis. The functional consequence of AR expression in kidney medulla was made clear when it was discovered that exposure of a line of renal papillary epithelial cells to increased extracellular osmolarity stimulated an increase in intracellular sorbitol [65]. In addition, ablation of AR gene in mice results in defect in kidney function. The mice become unable to concentrate their urine to normal level [62]. However, it is not clear why the absence of AR leads to a defect in water resorption, as sorbitol constitutes only 2% of the total osmolality of mouse kidney [66]. No such phenotype has been reported when animal models from other species (rat) are treated with AR inhibitors. This suggests that either AR inhibitors are not sufficiently effective to reduce sorbitol levels enough to cause a defect in urinary concentration, or that other mechanisms, not present in the mouse kidney, are able to functionally compensate for the loss of AR activity.

The increased expression of AR under hyper-osmotic stress was subsequently reported in a variety of cells of non-renal origin, such as Chinese hamster ovary cells [67], cultured human retinal pigment epithelial cells [68], and human embryonic epithelial cells [69]. Transient transfection studies with 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 the transcription of AR gene [70-72]. Studies on the factors that interact with these response elements and augment the transcription of AR gene may provide insight into the regulatory mechanisms of the gene expression.

2. Detoxification

While AR is thought to play a major role in the synthesis of sorbitol as an osmolyte in the kidney medulla, its distribution among tissues unaffected by extracellular osmotic stress suggests an alternate metabolic role. In addition, the marked hydrophobic nature of the active site is unusual for an enzyme thought to be involved in the metabolism of aldo- sugars. It has been demonstrated that unusual for an enzyme thought to be involved in the addition, the marked hydrophobic nature of the active site is characteristic of enzymes that remove and detoxify toxic compounds such as reactive aldehydes derived from glucose or its oxidation products. Sorbitol is one of the osmolytes that balance the osmotic pressure of extracellular NaCl, fluctuating in accord once with urine osmolality [64]. The interpretation of these findings is that AR fulfills a role as an oxidative defense protein.

In a series of aldehyde substrates for human AR investigated isocorticosteroids [77] and isocaproaldehyde [78] both with $K_m$ values of approximately 1 µM or less, they are the best physiological substrates known to date. The next preferred substrates for AR may be aldehydes derived from biogenic amines [79] and methylglxyoxal, a toxic aldehyde produced nonenzymatically from triose phosphate and enzymatically from acetone/acetol metabolism [80]. 17α-hydroxyprogesterone [81] and 4-hydroxynonenal [82], a reactive aldehyde produced by oxidative damage to unsaturated fatty acids, are also excellent substrates for the enzyme with $K_m$ values of 20–30 µM. Another line of study demonstrated that 3-deoxy-glucosone, one of the cross-linking agents formed as intermediates in non-enzymatic glycation, is a good substrate for AR [83].

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 [84]. Both 3-deoxyglucosone and acrolein exhibited a similar range of $K_m$ values (40-80 µM) in the kinetic analysis [85]. Whereas glucose is one of the endogenous substrates for AR, comparison with other endogenous aldehydes indicates that glucose is a rather poor substrate with a $K_m$ value of 70 mM [86]. The interpretation of these findings is that AR in the adrenal gland and reproductive organs may normally participate in the synthesis and catabolism of steroids hormones, whereas it is involved in the metabolism of biogenic amines in the central nervous system. The enzyme may also act as an extrahepatic detoxification enzyme in various tissues. Thus, the significance of AR in the polyol pathway may be quite limited under non-diabetic conditions: it provides an osmolyte sorbitol in the renal medulla and supplies fructose as an energy source of sperm in the seminal vesicle.

3. Unique Tissue Distribution Pattern of AR

Recent investigations disclosed the unexpected distribution pattern of AR not only in different species but in tissues other than “target” organs of diabetic complications. In human tissues, it is quite abundant in the epithelial cells lining the collecting tubules in the renal medulla. Additionally, AR is also found in many other tissues such as seminal vesicles, retina, lens and muscle [87]. In mouse, AR mRNA was most abundantly expressed in the testis, whereas a very low level of the transcript was detected in the sciatic nerve and lens [88]. These results suggest that mouse AR 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 inflammation and ischemia [74]. Additionally, it has been shown that cells resistant to oxidative stress express higher levels of AR than the corresponding sensitive cells and that the inhibition of AR increases the sensitivity of these cells to cytotoxic aldehydes [75]. This view is further reinforced by the observation that several structurally different phospholipids aldehydes are efficiently reduced by the enzyme AR, suggesting that AR may be an important component of mechanisms that remove and detoxify these aldehydes when they are generated in oxidized lipids [76]. It could be concluded that AR fulfills a role as an oxidative defense protein.

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the enzyme transcript in these “target” organs of diabetic complications [21]. Consistent with these findings is the absence of cataract formation during the course of hyperglycemia in mouse [58], in contrast with the finding in rat, the first experimental model of sugar cataract formation [1]. Immunoblot and immunohistochemical analyses in rat tissues further showed high levels of AR protein in the adrenal gland and various reproductive organs, including the granulosa cells of rat ovary [89]. Of particular interest is the fact that cyclic changes in the expression and localization of AR were observed in rat ovary during the estrous cycle [90]. These changes in the enzyme expression were indicated to be under hormonal control, and the study suggests another functional role of AR in the female reproductive organ, which can be deranged under diabetic conditions.

D. Aldose Reductase in Glucose Toxicity

Aldose reductase is the first, and rate-limiting enzyme of the polyol pathway [19]. Under euglycemic conditions, AR plays a minor role in glucose metabolism, however, during diabetes, its contribution is significantly enhanced [1]. Increased AR activity by hyperglycemia has been proposed to greatly influence the development and progression of secondary diabetic complications (retinopathy, neuropathy and nephropathy) [51]. Retinal capillary pericytes contain the enzyme AR and the accumulation of excess sugar alcohol catalyzed by AR in pericytes, has been linked to their degradation [4].

1. Effect of Accelerated Polyol Pathway

Increased activity of AR and the polyol pathway has been suggested to be underlying biochemical cause of secondary diabetic complications [19]. The induction of osmotic stress from the excess intracellular accumulation of sugar alcohol (polyol) can lead to altered membrane permeability and subsequently to biochemical changes that result in the initiation of cellular lesion (polyol osmotic theory) [8]. In addition, it has been shown that transgenic over expression of AR gene in mice causes sorbitol accumulation in target tissues following diabetes induction [91]. Moreover, the role of AR in the pathogenesis of diabetic complications is further supported by the evidence showing that inhibition of the enzyme prevents and/or delays the development of diabetic cataracts, neuropathy, nephropathy and retinopathy [1]. These observations suggested a major role for polyol pathway in glucose toxicity proven to be responsible for secondary diabetic complications. However, in cell culture studies, hyperglycemia induces progressive resistancete of the enzyme to inhibition [92]. AR isolated from diabetic humans or animals is more resistant to pharmacological inhibition than that from normal euglycemic tissues [46].

In the retina, the localization of AR in retinal microvessels was demonstrated in various animal species including human [8, 93, 94]. In hyperglycemia, glucose may accumulate to supernormal intra-cellular level as a result of an increased uptake and decreased efflux [95]. The glucose accumulation in the retina in diabetic patients is greater than that in normal retina [96]. Enhanced AR activity is a mechanism for cardinal manifestation of diabetic retinal microangiopathy common to date in human [6]. Additionally, it has been reported that increased prevalence of retinopathy correlates with increased levels of erythrocytes AR activity [97]. Treatment with AR inhibitors was shown to prevent capillary basement membrane thickening, the early structural lesion observed in the retina [98-100]. Recently, it has been showed that human retina expressed sorbitol dehydrogenase (SDH) activity as indicated by increased fructose level after 24 and 48 hrs incubation of retinal cells in high glucose (unpublished observation). Therefore, fructose is the end product of polyol pathway in the retina. Because fructose and its metabolites fructose-3-phosphate and 3-deoxyglucosone are more potent non-enzymatic glycating agent than glucose, therefore, flux of glucose through the polyol pathway would increase advanced glycation end product (AGE) formation [101]. In the ocular lens, the accumulation of polyol induces hyperosmotic swelling and deranges the cell membrane, resulting in the leakage of amino acids and glutathione to provoke cataract formation [102]. 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 [103], whereas more than 30% of glucose is metabolized through this pathway under hyperglycemia [104]. The increased flux of glucose through this pathway and consequent expenditure of cofactors for AR (NADPH) and sorbitol dehydrogenase (NAD+) leads to a redox state change and a cascade of interrelated metabolic imbalances. Glutathione reductase and nitric oxide (NO) synthase are substantially affected 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 [101]. Indeed, increased susceptibility to H2O2 along with a reduced level of glutathione was reported in the endothelial cells cultured in high glucose medium [105]. 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 [106,107].

2. 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. Among the well-documented factors are activation of protein kinase C [108, 109], enhanced nonenzymatic glycation [110], and augmentation of oxidative stress [101]. Some of these are postulated to be correlated with each other. Both increased AR activity and oxidative stress have been implicated in the pathogenesis of diabetic complications [111]. The important role of two mechanisms in diabetic retinopathy is supported biochemical [112], functional [113] and biological [114] abnormalities in the retina. Both AR inhibitors and antioxidants prevent formation of retinal pericyte ghosts and acellular capillaries [112], increased vascular permeability [115] and decreased blood flow. 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 is attenuated because of reduced glutathione availability and inactivation of superoxide dismutase. Vascular dysfunction and the resulting derangement in tissue perfusion under diabetic conditions induce ischemia and reperfusion process, which further generate oxygen free radicals [116]. On the other hand, it has been generally accepted that advanced glycation end product participates in the production of oxygen free radicals [117]. Inactivation of superoxide dismutase in diabetes was demonstrated to result from glycation of the two lysine residues on the enzyme protein [118]. 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. 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. Obrosova et al., [119] indicated a major contribution of AR to high glucose-induced oxidative stress in retinal epithelial cells and showed that fidarestat (AR inhibitor) can arrest hyperglycemia-induced reactive oxygen.

Diabetes associated phenomena such as increased retinal AR activity, oxidative stress and increased vascular permeability are interrelated. AR triggers the whole cascade by causing oxidative stress, which in turn leads to the overexpression of vascular endothelial growth factor (VEGF) [120] responsible for increased vascular permeability [121]. Indeed, all three components of this cascade have been found preventable by an AR inhibitors treatment [119].

Activation of protein kinase C was reported in vascular smooth muscle and endothelial cells after the exposure to hyperglycemia. The rise in NADH/NAD+ ratio via enhanced polyol pathway may also facilitate the diacylglycerol (DAG) synthesis by increasing the availability of dihydroxyacetone phosphate as well as favoring its reduction to glyceraldehyde 3-phosphate, the intermediates of DAG synthesis [122]. The increased protein kinase C activity attenuates 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 pump activity that regulates intracellular pH, cell growth, and differentiation and also augments expression of various matrix proteins such as fibronectin, and type IV collagen [109]. 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. A specific inhibitor for the β isoform of protein kinase C was shown to ameliorate vascular dysfunctions in diabetic rat [123].

3. Aldose Reductase and Thickening of Basement Membrane

Diabetic retinal complications result from retinal capillaries functional and morphological alterations: increased permeability to albumin and macromolecules, vascular dysfunction, loss of pericytes and basement membrane thickening.

Capillary basement membrane thickening and hypertrophy of extravascular matrix are common features of diabetic microvascular complications. The link between high plasma glucose levels and tissues damage is due in part to the formation and accumulation of advanced glycation end products (AGEs) in tissues [124]. AGEs accumulate in extracellular matrix proteins as a physiological process during aging [125]. However, this accumulation happens earlier, and with an accelerated rate in diabetes mellitus than in non-diabetic individuals [126]. Increased serum and tissue levels of AGEs due to reduced removal by kidney have been evidenced in end stage renal failure [127] and are more important in diabetic than non-diabetic patients [128]. A highly significant correlation has been shown between the importance of AGEs deposits and the severity of diabetic complications [129]. In vitro and in vivo studies have indicated that AGEs induce irreversible cross-links in long-living matrix structural proteins such as collagen [129]. AGEs are implicated in the basement membrane thickening through these alterations, via a reduction in susceptibility of matrix proteins to proteolytic degradation [130]. These architectural changes also alter the functional properties of the basement membrane including permeability. Advanced glycation of proteoglycans induces a decrease in the electronegative charges [131] and therefore modifies selective filtration properties of the basement membrane. These alterations lead to macular edema secondary to the leakage of macromolecules. Capillary closures are responsible for non-perfused areas (ischemic retinopathy), which induce the secretion of vascular endothelial growth factor (VEGF) and the development of neo-vessels (proliferative retinopathy). Evidence of this role relies on the results of studies indicating that the deleterious effects of AGEs on retinal capillary pericytes and endothelial cells are inhibited by AGEs receptors antibodies.

E. A Potential Target for the Prevention of Diabetic Complication

Aldose reductase has been implicated in the etiology of diabetic complications. A variety of compounds have been observed to inhibit AR. Orally active inhibitors of the enzyme have been investigated for many years. Although several of these compounds have progressed to the clinical level, only one such drug is currently on the market [132]. Due to the limited number of a valuable drug for the treatment of diabetic complication, a number of rational approaches for the discovery of AR inhibitors have under been taken since the determination of the 3-dimensional structure of the enzyme. There are a variety of structurally diverse AR inhibitors.

1. Clinical Trials of AR Inhibitors

El-Kabbani et al. [2] showed that the incidence of retinopathy was significantly influenced by mean blood glucose levels and study participants practicing intensive as compared to conventional glycemic control experienced a marked reduction in the incidence and progression of diabetic retinopathy. The protective effect was less noticeable if intensive control was instituted after some progression occurred. Similar results were found in the study of galactosemic dogs, which showed that correction of
hyperglycemia through removal of dietary galactose did not stop the progressive appearance of retinal abnormalities associated with diabetic retinopathy [133]. Taken together, these studies emphasize that retinopathy followed a long-term pathogenesis and that once initiated, pharmacological efforts to interfere are far less likely to be successful than prophylactic treatment prior to onset of early and perhaps, irreversible tissue changes. Therefore, even if the therapeutic basis for AR inhibition is valid, efficacy for a given drug might be impossible to establish if the clinical study design does not take into consideration known risk factors for retinopathy such as duration of diabetes, rigor of glycemic control and existence of early changes in retinal vasculature. It is encouraging that new inhibitors are becoming available [134, 119]; Some with novel structures free from the hydantoin nucleus found in inhibitors such as sorbinil, better tissues penetration and activity than the carboxylic acid inhibitors and higher selectivity against AR [135]. A similar rationale applies for AR inhibitors studies to establish efficacy toward diabetic neuropathy. In addition to these potential design flaws in prior clinical trials, failure to demonstrate inhibitor efficacy may be related to poor pharmacokinetic profiles of the investigated compounds. For example, inadequate nerve penetration almost certainly contributed to the failure of ponalrestat in clinical trials for diabetic neuropathy. In addition, unexpected toxicity was a factor leading to the termination of clinical trials of sorbinil and tolrestat [136]. However, a promising effect of aldose reductase inhibitor on nerve conduction velocity was reported. 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 [137]. Subsequently, numerous clinical studies were carried out to evaluate the efficacy of sorbinil. However, the major adverse reaction of sorbinil was a hypersensitivity reaction in the early weeks of therapy, which is similar to that seen with other hydantoins. The efficacy of another class of inhibitor, tolrestat, was modest in diabetic patients already symptomatic of neuropathy [138], although the progress of mild diabetic autonomic and peripheral neuropathy could be halted [139]. 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 [140].

2. Classes of AR Inhibitor

A list of the most commonly available inhibitors contains either a cyclicimide groups, such as spirohydantoin group or spirosuccinimide group, an acetic acid moiety. The best known of the spirohydantoin-containing compounds, sorbinil, has been thoroughly analyzed by structural analysis of the AR and NADPH, and several clinical studies. Other examples of these groups include fidarestat and its stereoisomers. There are numerous compounds that incorporate the acetic acid moiety, and they include tolrestat, ponalrestat (stabil) and zopolrestat. It is noted that the cyclicimide or acetic acid moieties bind to an essentially hydrophilic area of the active side of AR, which contains the Tyr-48, His-110 and Trp-111 residues.

Another common feature among the various inhibitors is the presence of one or more aromatic groups, which may include phthalazinyl group (ponalrestat), a naphthyl group (tolrestat), a benzothiazole group (zopolrestat), a 2' -thioxo-1,3-thiazolan-4-one group (epalrestat) and a halogenated benzylic group (ponalrestat). These aromatic groups bind in the hydrophobic pocket of AR, Trp-111, Phe-122 and Leu-300 residues, either through hydrogen bonding or hydrophobic contact.

Inhibitors containing the cyclicimide or carboxylic groups exhibit similar in vitro but different in vivo activities [141]. The carboxylic acid-containing inhibitors have lower in vivo activity, which has been attributed to relatively lower pKa values, thus causing ionization at physiological pH and an inability to traverse cell membranes. Cyclicimides have higher pKa and are only partially ionized at physiological pH, thus being able to pass through cell membranes and have better pharmacokinetic properties. Sorbinil possessed all these attributes, but its development as a therapeutic agent was halted due to hypersensitive reaction [142].

The flavonoids (2-phenyl-4H-1-benzopyran-4-one) are also good inhibitors of AR [143] but do not contain either the carboxylic acid or cyclicimides moieties. This class of inhibitors, both naturally occurring and synthetic, has higher pKa values than the carboxylic acid [144] and also has antioxidant properties [145], which prevent 4-hydroxy-2,3-trans-nonenal (HNE)-induced cataract formation.

A newer class of AR inhibitors is the phenylsulfonylnitromethanes [146], which exhibited potent activity against AR and some of which also showed irreversible inhibition.

The departure of the NADP+ from the enzyme is believed to be the rate-determining step and this is when the current AR inhibitors inhibit enzyme action. It was shown that the inhibitors that bind to hydrophobic pocket were better AR inhibitors. Such knowledge has been utilized in the design of potent and specific inhibitors of AR. However, modeling studies have shown that inhibitors specific to AR should interact with the C-terminal residues by binding to specificity pocket [41]. The hydrogen-bonding interactions between the inhibitors and active site residues (Tyr-48, His-110, Trp-111) oriented the inhibitor in the active site.

3. Variable Levels of Aldose Reductase in Diabetic Patients

Substantial variations in the levels of AR expression in various tissues exist among individuals with or without diabetes. Marked variability in AR activity was reported for enzyme preparations isolated from human placentas [86]. AR purified from erythrocytes exhibited a nearly three-fold variation in activity among diabetic patients [147]. 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 AR in numerous diabetic subjects. In the previous studies, investigators examined variations in AR by isolating the enzyme from placenta or erythrocytes and assaying its activity [86,147]. The isolation of the enzyme was necessary because of the presence of other structurally related members of aldo-keto reductase family, particularly aldehyde
reduce, in crude tissue preparations. These enzymes share overlapping substrate specificity with AR. A newly developed immunoassay method using a specific antibody directed against AR could circumvent such difficulties [148]. The amount of the enzyme determined by the immunoassay highly correlates with the activity of AR isolated from the erythrocytes of the same individuals [149]. By using this assay method, the association between the AR level in the erythrocyte, and various clinical parameters determined in patients with non-insulin-dependent diabetes mellitus (NIDDM) were investigated. 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 (HbA1c) 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 AR may affect the susceptibility and prognosis of diabetic retinopathy [8,150]. 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 [151]. The erythrocyte AR level was significantly higher in those patients showing overt neuropathy compared with those without demonstrable neuropathy. A higher level of AR is one of the independent risk factors for overt neuropathy. Accordingly, these results support the hypothesis that a difference in the level of AR is responsible for the susceptibility of diabetic patients to toxic effects of glucose. The activity of AR fractionated from the erythrocytes was reported to be significantly higher in IDDM patients with complications compared with those showing no signs of complications [152]. Increased levels of AR protein were also demonstrated by immunoblot analysis in the mononuclear cells isolated from IDDM patients with apparent diabetic complications [153]. The level of AR 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 [151]. In this study, the enzyme level remained unchanged irrespective of improved or stably high HbA1c levels during the follow-up period. These 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 AR observed in diabetic patients may be genetically determined. To explore this possibility, two regions on the aldose AR relevant to the enzyme expression were examined: the promoter region containing a TATA box [154], and the region containing identified osmotic response sequences [72]. However, in the DNA sampled from 700 NIDDM patients with different enzyme levels in the erythrocyte, there was no change in either of these regions associated with differences in the expression of AR levels [155]. Thus, the reason for the variable expression of AR 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 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 AR 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 AR 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 AR 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 AR 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.

CONCLUSION

Diabetes mellitus is recognized as a leading cause of new cases of blindness and is associated with increased risk for painful neuropathy, heart diseases and kidney failure. Many theories have been advanced to explain mechanisms leading to diabetic complications including accelerated protein glycation, altered signaling involving PKC, excessive oxidative stress and stimulation of glucose metabolism by the polyol pathway. It has been demonstrated that polyol pathway is the major source of diabetes induced oxidative stress, as AR activity depletes its co-factors NADPH, which is required for glutathione reductase to regenerate GSH. In addition to the formation of fructose and its metabolites, which are potent non-enzymatic glycatng agents, AR increases AGE. Therefore, designing and screening specific inhibitors for AR is becoming the therapeutic strategies that have been proposed to delay or prevent diabetic complications.

The tertiary structure of aldose reductase, including the active site and the interaction with inhibitors of diverse chemical structures has been resolved. However, much still remains to be elucidated regarding the pathophysiological significance of the enzyme and the regulatory mechanisms of AR expression in various human tissues. In diabetic animal models, promising effects of AR inhibitors were demonstrated. However, most of the clinical trials carried out so far, produced rather modest or disappointing effects of the inhibitors on the functional and morphological improvements in diabetic retinopathy. 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. Secondly, the relative abundance of the aldo-keto reductase family, such as aldehyde reductase, which is colocalized in human tissues and later may interfere with the action of inhibitors to suppress AR. Lastly, the efficacy of AR 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 pathogenic mechanisms of diabetic complications. If multiple
mechanisms are involved in the pathogenesis of diabetic complications, the extent of the effectiveness of AR 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 AR in “target” tissues.

REFERENCES


