0163-769X/05/$20.00/0
Printed in U.S.A.

Endocrine Reviews 26(3):380–392

doi: 10.1210/er.2004-0028

Role of Aldose Reductase and Oxidative Damage in Diabetes and the Consequent Potential for Therapeutic Options

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Aldose reductase (AR) is widely expressed aldehyde-metabolizing enzyme. The reduction of glucose by the AR-catalyzed polyol pathway has been linked to the development of secondary diabetic complications. Although treatment with AR inhibitors has been shown to prevent tissue injury in animal models of diabetes, the clinical efficacy of these drugs remains to be established. Recent studies suggest that glucose may be an incidental substrate of AR, which appears to be more adept in catalyzing the reduction of a wide range of aldehydes generated from lipid peroxidation. Moreover, inhibition of the enzyme has been shown to increase inflammation-induced vascular oxidative stress and prevent myocardial protection associated with the late phase of ischemic preconditioning. On the basis of these studies, several investigators have ascribed an important antioxidant role to the enzyme. Additionally, ongoing work indicates that AR is a critical component of intracellular signaling, and inhibition of the enzyme prevents high glucose-, cytokine-, or growth factor-induced activation of protein kinase C and nuclear factor-κ-binding protein. Thus, treatment with AR inhibitors prevents vascular smooth muscle cell growth and endothelial cell apoptosis in culture and inflammation and restenosis *in vivo*. Additional studies indicate that the antioxidant and signaling roles of AR are interlinked and that AR regulates protein kinase C and nuclear factor-κB via redox-sensitive mechanisms. These data underscore the need for reevaluating anti-AR interventions for the treatment of diabetic complications. Potentially, the development of newer drugs that selectively inhibit AR-mediated glucose metabolism and signaling, without affecting aldehyde detoxification, may be useful in preventing inflammation associated with the development of diabetic complications, particularly micro- and macrovascular diseases. (*Endocrine Reviews* 26: 380–392, 2005)

I. Introduction: Aldose Reductase and Diabetic Complications

ALDOSE REDUCTASE (AR) is a monomeric reduced nicotinamide adenine dinucleotide (NAD) phosphate (NADPH)-dependent enzyme and a member of aldo-keto reductase superfamily. The enzyme was described in 1956 by Hers (1) as a glucose-reducing activity. It was subsequently reported by van Heyningen (2) that high levels of AR activity are present in the rat lens and that during diabetic and galactosemic cataractogenesis, AR-derived polyols—sorbitol and galactitol—accumulate in the ocular lens. Building on this observation, Kinoshita *et al.* (3) and Varma and Kinoshita (4) demonstrated that treatment with pharmacological inhibitors of AR ameliorated cataractogenesis in diabetic rats and galactose-exposed rabbits. Based on these observations it was proposed that accumulation of sorbitol in the lens, due to AR-catalyzed reduction of glucose, causes osmotic swelling resulting in ionic imbalance and protein insolubilization leading to cataractogenesis (5–7). A similar sequence of events could also account for hyperglycemic injury associated with diabetic retinopathy, nephropathy, and neuropathy (Fig. 1).

The osmotic hypothesis that diabetic complications are due to sorbitol accumulation in tissues has engendered extensive investigations over the last three decades. Several drugs with varying AR-inhibiting efficacy (*e.g.*, sorbinil, statil, tolrestat, and zopolrestat) have been synthesized and tested (8–11). Initial trials in animal models showed significant protection against diabetic complications (12–14). AR inhibitors, in addition to preventing diabetic and galactosemic cataracts (3, 4), ameliorated some of the features of diabetic nephropathy (15, 16) and neuropathy (17–19). However, clinical trials with AR inhibitors have yielded uncertain results, in part due to the high nonspecific toxicity of this class of drugs.

In the mid-1980s we demonstrated that most of the inhib-

First Published Online April 6, 2005

Abbreviations: AR, Aldose reductase; DAG, diaceylglycerol; DHN, 1,4-dihydroxy-2-nonene; FGF, fibroblast growth factor; GSNO, nitrosoglutathione; GSSG, oxidized glutathione; HNE, 4-hydroxynonenal; NAD, nicotinamide adenine dinucleotide; NADPH, reduced NAD phosphate; NF-κB, nuclear factor-κ-binding protein; NO, nitric oxide; PKC, protein kinase C; PLC, phospholiase C; VSMC, vascular smooth muscle cells.

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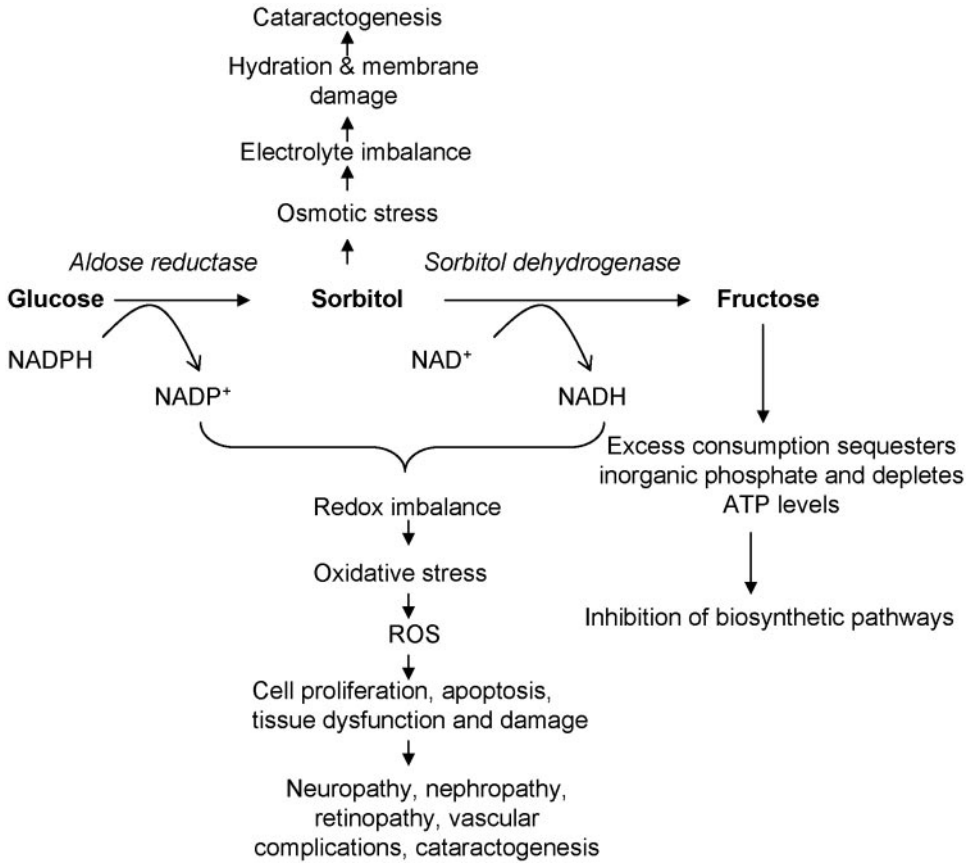

FIG. 1. Involvement of polyol pathway in diabetic complications. During hyperglycemia, reduction of glucose to sorbitol by AR constitutes the first and the rate-limiting step of the polyol pathway that converts glucose to fructose via sorbitol dehydrogenase (SDH). In this pathway both NADPH and NAD^+ are consumed as cofactors for the enzymes AR and SDH. Osmotic stress due to accumulation of sorbitol and oxidative stress due to changes in the ratio of $NADPH/NADP^+$ and reduced NAD (NADH)/NAD^+ are the major cause of various complications of secondary diabetes. ROS, Reactive oxygen species.

itors synthesized by pharmaceutical companies were not selective for AR but also inhibited other members of the aldo-keto reductase superfamily such as aldehyde reductase (20). In addition, we also demonstrated that increased osmotic pressure due to accumulation of polyols in galactosemic and diabetic cataractogenesis could not be the main cause of cataractogenesis (21–23). Antioxidants such as butylated hydroxytoluene and 6-hydroxy-2,5,7,8-tetramethenyl-chroman-2-carboxylic acid (Trolox) prevented diabetic cataractogenesis in rats and significantly attenuated lens opacity in galactose-fed rats even though the polyol levels in the lens were extremely high (80–90 mM), indicating that polyol accumulation *per se* is not sufficient for cataractogenesis and that other metabolic changes accompanying AR activation may be critical and important modulators of the cataractous effects of hyperglycemia and diabetes (Fig. 2).

Because AR utilizes NADPH to catalyze glucose reduction, we, as well as other investigators, suggested that tissue injury associated with high glucose may be due, in part, to increased NADPH utilization by the reduction of glucose by AR (24, 25). In the presence of normal glucose (5.5 mM), AR-catalyzed reduction represents less than 3% of total glucose utilization, whereas in the presence of high glucose (20 mM), more than 30% of the glucose is used by AR (26), suggesting that the profound increase in the AR-catalyzed reductive pathway may impose a significant strain on NADPH supply. Because NADPH is used for several critical reductive metabolic steps, such as the detoxification of reactive oxygen species and hydroperoxides (*e.g.*, by the glutathione reductase/glutathione peroxidase system), a large drain on the NADPH pool could compromise the ability of the cell to protect itself from oxidative stress.

Another reason for the inconsistent effects of AR inhibitors may relate to posttranslation modification of the enzyme. As documented extensively in our previous publications (26–28), the oxidation state of a single cysteine residue located at the active site of the enzyme regulates both substrate and inhibitor binding and thus, given the extensive oxidative changes in diabetes, it is likely that in diabetic tissues AR is

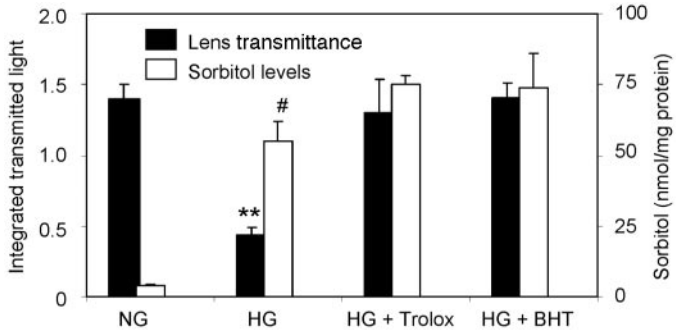

FIG. 2. Oxidative stress is the main contributor to diabetic cataractogenesis. Digital image analysis was performed on lenses cultured in normal glucose (5.5 mM; NG), high glucose (50 mM; HG) without or with antioxidants on d 8. Sorbitol content was measured by using gas chromatography. The data represent mean ± SD (n = 4). **, $P < 0.001$; #, P = nonsignificant in HG-treated group compared with NG-treated group. BHT, Butylated hydroxytoluene.

insensitive to inhibitors due to oxidative modification. In support of this view, we have demonstrated that AR in human erythrocytes exists in two forms, activated and unactivated, and in hyperglycemia total activity of AR increases (29). Multiple forms of AR have also been reported by other investigators (30, 31), and our *in vitro* data suggest that oxidants can induce a variety of changes in the catalytic activity of the enzyme, although which of these forms occur *in vivo* and how they are interconverted remains unclear. Nevertheless, the view that the hyperglycemia changes the sensitivity of AR to inhibition is further supported by the observation that prolonged culture of endothelial cells in high glucose progressively decreases the efficacy of sorbinil in preventing sorbitol accumulation (32).

In contrast to inhibitor data, newer molecular approaches have provided more unambiguous evidence for the involvement of AR in diabetic complications. In these studies transgenic overexpression of AR gene selectively in the lens was found to accelerate diabetic and galactosemic cataract formation in mice (33). Additionally, ubiquitous overexpression of the AR gene increased the rate of neuropathic changes in diabetic animals (34–36). Collectively, these data argue strongly that AR is an important component of high glucose-induced metabolic changes that underlie the development of secondary diabetic complications. Nevertheless, despite this evidence, the exact mechanism by which AR contributes to the development of diabetic complications remains unclear. Persistent increase in extracellular glucose levels induces pleiotrophic changes in metabolism that elicit polygenic responses (Fig. 3). Extensive changes in the activation of protein kinases and the accumulation of advanced glycation end products have been reported (37–39), although their relationships with modulatory influences on AR remain unclear. In principle, AR could contribute to both protein kinase activation and advanced glycation end product accumulation. In addition, AR could catalyze the formation of potent protein glycating agents and induce oxidative stress.

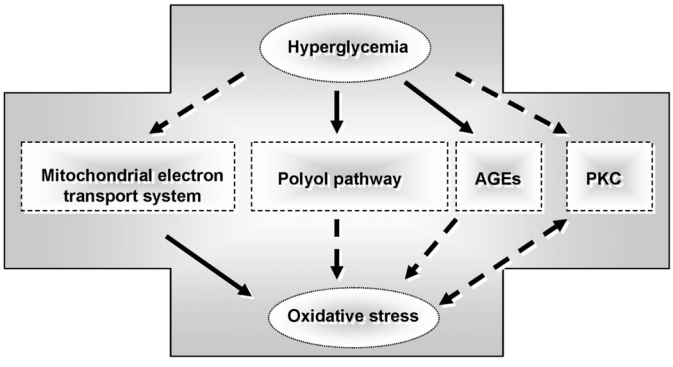

FIG. 3. Pathways of hyperglycemia-induced oxidative stress. Increased superoxide production in the mitochondria during hyperglycemia causes increased oxidative stress. During hyperglycemia the ratio NADPH/NAD^+ decreases due to excessive use of NADPH for the reduction of glucose to sorbitol. Consequently, the NADPH availability to maintain glutathione in the reduced form catalyzed by glutathione reductase decreases, which causes oxidative stress. Increase in advance glycation end products (AGEs) formation and binding of AGE to its cognate receptor (RAGE) also increases oxidative stress. Increased DAG formation in hyperglycemia activates PKC and subsequently induces oxidative stress via a PKC-dependent activation of NAD(P)H oxidase.

Reduction of glucose by AR leads to the formation of sorbitol, which, in some tissues, is further oxidized to fructose upon sorbitol dehydrogenase-catalyzed oxidation. The conversion of glucose to fructose (the "polyol pathway") results not only in the utilization of NADPH, but also NAD^+. As a result, increased activity of the polyol pathway during hyperglycemia could lead to a depletion of NADPH and accumulation of reduced NAD. This shift in the redox state of pyridine coenzymes recapitulates the metabolic phenotype of hypoxia and has been proposed to induce a state of pseudohypoxia resulting in hypoxia-like responses (40–42). Polyol pathway-mediated alterations in pyridine nucleotides have been linked to diverse metabolic changes such as the synthesis of nitric oxide (NO) and activation of protein kinases (39). Specifically, it has been proposed that the increase in NADH due to elevated polyol pathway activity could increase the synthesis of diacylglycerol (DAG) from dihydroxyacetone phosphate (43). DAG levels could also increase by stress that activates phospholipase C (PLC in Fig. 4). DAG is an essential activator of the classical and novel members of the protein kinase C family, and DAG-dependent activation of these kinases is thought to play a key role in mesangial expansion and smooth muscle cells proliferation induced by high glucose (44–46). Inhibitors of specific protein kinase C (PKC) isoforms are currently under clinical trial for the treatment of diabetic complications (47, 48).

II. AR and Antioxidant Protection

Due to its ability to reduce glucose, AR is involved in several tissue-specific metabolic pathways. For instance, in the kidney, AR participates in osmoregulation (49, 50) and, in seminal vesicles, in the generation of fructose (1). The

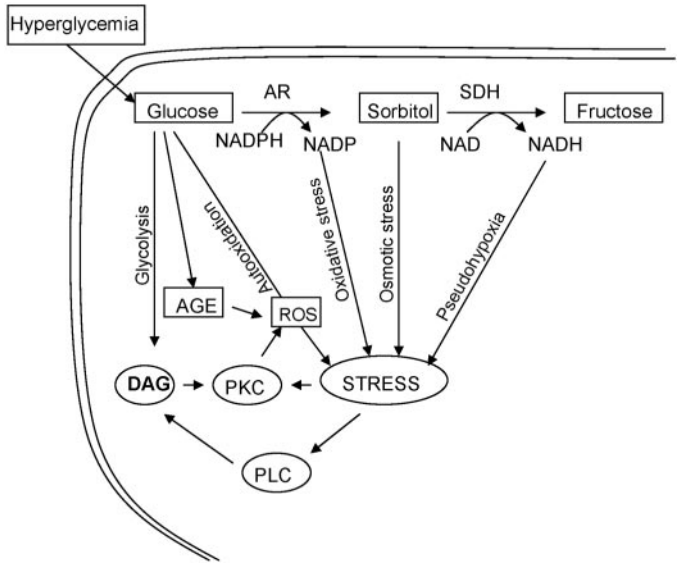

FIG. 4. Pathways of increased DAG in hyperglycemia. Increased stress conditions because of autooxidation of glucose, osmotic stress, oxidative stress, and hypoxia during polyol pathway hyperactivity can contribute to increase in DAG levels through a cascade of signals that activate phospholipase C (PLC) or directly through glycolysis. AGE, Advance glycation end product; ROS, reactive oxygen species; SDH, sorbitol dehydrogenase.

observation that AR and related proteins are up-regulated by fibroblast growth factor (FGF) and other mitogens suggests that AR may be involved in cell growth or growth factor-induced changes in cellular metabolism (51–61). Our studies show that *in vitro* homogenous AR catalyzes the reduction of a large series of saturated and unsaturated aldehydes with 10^3- to 10^4-fold higher efficiency than glucose (62). The enzyme is particularly efficient in reducing medium- to long-chain (C-6 to C-18) aldehydes such as those generated in high abundance during lipid peroxidation (63, 64). The enzyme also catalyzes the reduction of the glutathione conjugates of unsaturated aldehydes, in many cases with efficiency higher than that of the parent free aldehyde (65–67). The reduction of glutathione conjugates by AR may be a protective mechanism that may be useful in minimizing the reactivity of the aldehyde function unquenched by glutathiolation. In addition, our recent studies show that AR is also an efficient catalyst for the reduction of core aldehydes—or aldehydes generated in oxidized phospholipids (Refs. 65–70 and Fig. 5).

The role of AR in detoxification is supported by structural studies showing that the active site of AR lacks ionic residue characteristic of carbohydrate-binding proteins (71–76). Instead, x-ray analyses of AR crystals reveal a highly plastic and hydrophobic active site (73, 74). This site could potentially accommodate a wide range of structures if the aldehyde function were to orient itself appropriately between the two ionic active site residues, His-110 and Tyr-48, which participate in acid-base catalysis. The structure of the binary complex of the enzyme with $NADP^+$ also indicates a profound conformational change upon NADPH binding (72). It has been suggested that the energy released as a result of this interaction is used for stabilization of the transition state with little demand from energy stabilization due to substrate binding (77). Hence, as a result of high-affinity interaction with NADPH, AR functions as an unusually promiscuous aldehyde removase, features that seem to be critically required of a detoxification enzyme involved in the removal of a wide range of aldehydes and glutathione aldehyde adducts generated during lipid peroxidation.

Based on our substrate specificity studies showing high-affinity reduction of glutathione conjugates by AR (66, 67), we reasoned that the active site of the enzyme must conform to a specific glutathione-binding domain. Consistent with the presence of specific interactions between the amino acid residues of glutathione and the AR active site, we found that alterations in the structure of glutathione diminished the catalytic efficiency for the reduction of glutathione-aldehyde conjugates and that nonaldehydic conjugates of glutathione or glutathione analogs displayed active site inhibition (67). Molecular dynamics calculations suggest that the conjugates adopt a specific low-energy configuration at the active site (Fig. 6). Mutations of the active site residues identified by these calculations selectively decreased catalysis of the glutathione-aldehyde conjugates, without affecting reduction of the free aldehyde. The high specificity and selectivity with which the enzyme catalyzes the reduction of glutathione conjugates suggest that such conjugates may be *in vivo* substrates of the enzyme. Indeed, our metabolic studies with 4-hydroxynonenal (HNE) show AR-dependent reduction of its glutathione adduct in heart, smooth muscle, and erythrocytes (78–81).

Reduction of aldehyde-glutathione conjugates by AR may be of physiological significance, particularly under conditions of oxidative stress when other antioxidant mechanisms are overwhelmed. Under these conditions, AR, by reducing both free aldehydes and their glutathione conjugates, could promote efficient removal and detoxification of unsaturated aldehydes, which are the major electrophilic end products of lipid peroxidation. Thus, investigations into the structure and kinetics of AR point toward a general detoxification role of the enzyme, indicating that lipid aldehydes and their glutathione conjugates are physiological substrates of the enzyme and that glucose is perhaps an incidental substrate, which is used only when it is high in concentration or occasionally for tissue-specific metabolism.

Consistent with its role as a detoxification enzyme, AR has been found to be regulated by aldehydes generated from lipid peroxidation (64, 70, 82), thiol-reducing agents (83–88), metal ions (89–91), and NO (92–97). Tissue levels of AR are also increased under conditions of high oxidative stress such

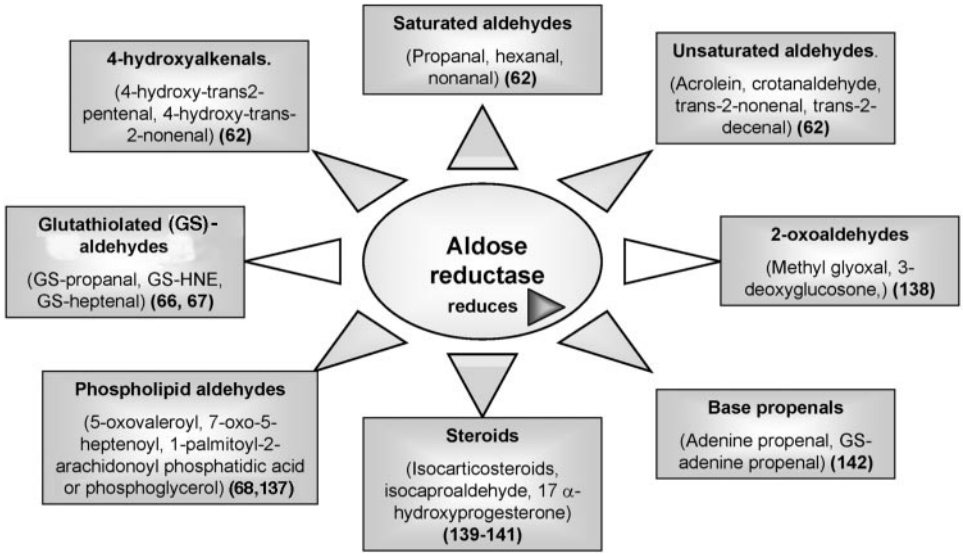

FIG. 5. Reduction of lipid aldehydes by aldose reductase. The *bold numbers in parentheses* represent reference numbers.

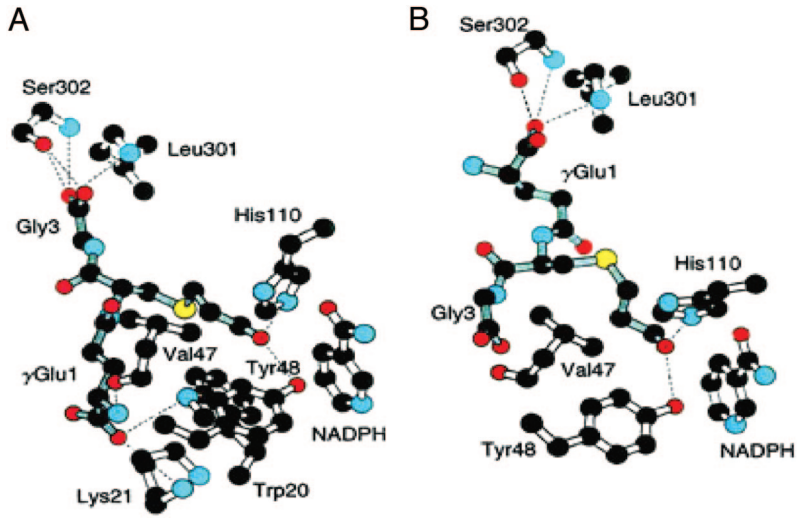

FIG. 6. Molecular modeling showing two possible orientations of glutathione-propanal bound to AR. Orientations 1 and 2 are shown in panels A and B, respectively. Carbon, nitrogen, sulfur, and oxygen atoms are colored *gray*, *blue*, *yellow*, and *red*, respectively. Potential hydrogen bonds are indicated with *dashed lines*. [Reproduced with permission from B. L. Dixit *et al.*: *J Biol Chem* 275:21587–21595, 2000 (67).]

as iron overload (98), alcoholic liver disease (99), heart failure (100), and vascular inflammation (53, 101). Moreover, AR is also specifically up-regulated during vasculitis, specifically in the areas of high HNE formation, and inhibition of AR increases the concentration of free HNE and protein-HNE adducts, accompanied by a 3-fold increase in the number of apoptotic cells (102). Our studies show that AR is also up-regulated in the heart by brief episodes of ischemia (103). This increase in AR was prevented by inhibitors of PKC and NO synthase, indicating that the up-regulation of AR is regulated by cardioprotective signaling associated with the late phase of ischemic preconditioning. Furthermore, a posttranslational activation of AR during myocardial ischemia has been reported (104). The functional significance of the increase in AR in the ischemic heart is underscored by the observation that inhibition of AR abolishes the cardioprotective effects of late preconditioning (105–107). Collectively, these data provide support to the view that AR is a component of an antioxidant defense mechanism, which protects tissues from the harmful effect of lipid peroxidation products such as HNE that accumulate during conditions of oxidative stress such as ischemia and inflammation.

Although HNE and related aldehydes are metabolized by several different pathways, metabolism via AR could represent a true detoxification mechanism. In most cells, HNE and related aldehydes readily form glutathione conjugates; however, the formation of glutathione-HNE itself may not be sufficient for detoxification. The glutathione-aldehyde conjugates are toxic. They induce DNA damage and stimulate radical formation (108–110). Therefore, reduction of the glutathione-aldehyde conjugates by AR may be necessary to substantially annul the reactivity of the conjugate and to decrease free radical generation. Nonetheless, the mechanisms by which AR-dependent aldehyde metabolism regulates the development of diabetic complications remain unclear, and the contribution of AR to cell growth and antioxidant defense requires further elucidation. In particular, it remains to be clarified how AR expression and activity are regulated and which components of intracellular signaling are regulated by AR.

III. Redox Regulation of AR Activity

As discussed above, a component of the hyperglycemic injury is due to an increased flux of glucose through the polyol pathway because the inhibition of AR prevents some of the diabetic complications. Although inefficacy of AR inhibitors could be due to their nonspecificity and hypersensitivity of selected individuals, the limited long-term efficacy of the AR inhibitors could be attributed to the posttranslational modification in AR that alters ligand binding and catalysis. In our earlier studies we found that AR isolated from individuals with different levels of hyperglycemia displayed altered kinetic properties and was less sensitive to hydantoin inhibitors such as sorbinil compared with the enzyme isolated from the tissues of normoglycemic subjects (29). Similar changes were observed at various stages of purification of AR in the absence of a reducing reagent such as dithiothreitol or β-mercaptoethanol from the tissues of normal subjects or from recombinant bacteria overexpressing AR (30, 31). Both the Michaelis-Menten constant (K_m) and inhibition constant (K_i) values of the enzyme significantly increased upon its *in vitro* thiol modification by HNE (64, 82), nitrosoglutathione (GSNO) (92), and oxidized glutathione (GSSG) (83).

The high sensitivity of AR to oxidants such as H_2O_2 and NO was attributed to a highly reactive cysteine (Cys-298) residue present at the active site of the enzyme (88). Oxidants such as H_2O_2 cause enzyme inactivation. Also glutathiolation of the Cys-298 results in a significant inactivation of the enzyme (83, 88, 92). However, depending on the conditions of the reaction and the nature of the NO donor used, AR is either S-thiolated (inactivated enzyme) or S-nitrosated (activated enzyme). On the basis of these observations, we hypothesized that NO regulates the intracellular activity of AR and consequently the flux of glucose via the polyol pathway (92–98). Because, in hyperglycemia, NO synthesis is significantly less compared with normoglycemic subjects, it has been reasoned that the AR activity may be up-regulated in diabetic tissues (111, 112).

Cardiovascular complications are one of the major causes

of mortality in patients with prolonged diabetes (113). The role of AR in cardiovascular complications, especially in atherosclerosis (100), restenosis subsequent to balloon injury (53, 57), and cardiac preconditioning (103–106), has been extensively investigated. Vascular endothelial cells are the main source of NO for vascular smooth muscle cells because in the vasculature, NO synthase is present mainly in the endothelium (114). The NO secreted by endothelial cells could readily form GSNO with glutathione that is abundant in vascular smooth muscle cells (VSMC). The GSNO formed could, in turn, readily S-glutathiolate AR at Cys-298 (92). Although GSSG (50–100 μM) also S-glutathiolates AR, it is unlikely that the physiological source of S-glutathiolation of AR (or other proteins) could be GSSG because the concentration of GSSG in cells rarely exceeds 20–25 μM. Because S-glutathiolation inactivates AR, under normoglycemic conditions, it appears likely that a significant fraction of AR in vascular tissues is present in an inactive form, whereas in hyperglycemia a decrease in $NADPH/NADP^+$ ratio and other factors would decrease NO and the AR would be in the active form. Indeed, we have demonstrated that, in diabetic rat aorta, both AR activity and sorbitol levels are more than 20-fold higher than nondiabetic aorta (95). Daily ip injections of NO synthase inhibitor, N^G-nitro-L-argininemethyl ester or application of nitroglycerin patch significantly increased the activity of aorta AR as well as sorbitol formation in both nondiabetic and streptozotocin-diabetic rats. On the other hand, daily injections of NO synthase substrate, L-arginine, significantly decreased AR activity and sorbitol content in aortas of both diabetic and nondiabetic rats. Similar results were obtained when aortic rings from normal and diabetic mice were incubated with L-arginine and N^G-nitro-L-argininemethyl ester, whereas, both NO synthase substrate and inhibitor had no effect on AR activity or sorbitol content of aorta from endothelial NO synthase-null mice (97). Interestingly, it was observed that S-glutathiolation of AR in VSMC by NO donors such as GSNO, which inactivates the enzyme, is reversible. Thus, the changes in glucose levels that alter the levels of NO would change the AR activity, sorbitol levels, and related effects in diabetic subjects. Hence, in addition to glycemic control, NO donors or drugs that increase NO levels could represent one treatment modality for the prevention or treatment of diabetic complications. Because hyperglycemia causes increased generation of reactive oxygen species by autooxidation of glucose and other metabolic pathways, antioxidants would also have beneficial effects. Diabetic complications such as cataractogenesis, retinopathy, and cardiovascular effects have been shown to be ameliorated by antioxidants such as butylated hydroxytoluene, butylated hydroxyanisole, Trolox, ascorbate, vitamin E, *N*-acetyl cysteine, pyruvate, *etc.* (115–120).

IV. Regulation of Intracellular Signaling by AR

Diabetes is a major risk factor for the development of cardiovascular disease. It is associated with a 2- to 4-fold higher risk of cardiovascular disease, and it accelerates the progression and increases the severity of atherosclerotic lesion formation in peripheral, coronary, and cerebral arteries (121–125). Moreover, diabetics have a higher propensity for restenosis after percutaneous transluminal coronary angioplasty (126). Even though coronary stenting significantly reduces restenosis, diabetes remains a powerful predictor of in-stent restenosis (127). Processes that lead to an increase in smooth muscle cell growth in diabetic as well as nondiabetic restenotic vessels have not been identified but, given the observation that high levels of protein-HNE adducts are associated with proliferative vascular lesion, it appears that products of lipid peroxidation, such as HNE, play a significant role in modulating the growth of vascular lesion. This is supported by our observation that treatment with low concentrations (<2 μM) of HNE increases VSMC growth in culture, although at higher concentrations HNE induced apoptotic cell death (53). To test the role of HNE in vascular proliferation, we determined how changes in its metabolism via AR would affect its mitogenic activity. Surprisingly, we found that inhibition of AR prevented VSMC growth in culture (53). Serum-starved VSMC cultured in 1 μM HNE showed increased proliferation compared with cells cultured without HNE, as determined by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay and cell count, whereas under similar conditions, HNE caused apoptosis in vascular endothelial cells and lens epithelial cells (Fig. 7). Both proliferation and apoptosis were attenuated by inhibiting AR using two structurally different AR inhibitors, sorbinil and tolrestat, and also by using antisense AR or small interfering RNA. In addition to HNE-stimulated growth, smooth muscle cells growing in response to serum FGF, TNFα, or high glucose were also sensitive to AR inhibitors (128). Moreover, inhibition of AR also prevented smooth muscle cell growth *in vivo*: we balloon-injured normal and diabetic rat carotid artery and followed restenosis by quantifying the neointima formation (101). In both normal and diabetic rats, AR inhibitor significantly (45%) prevented neointimal hyperplasia (Fig 8). Interestingly, the neointimal hy-

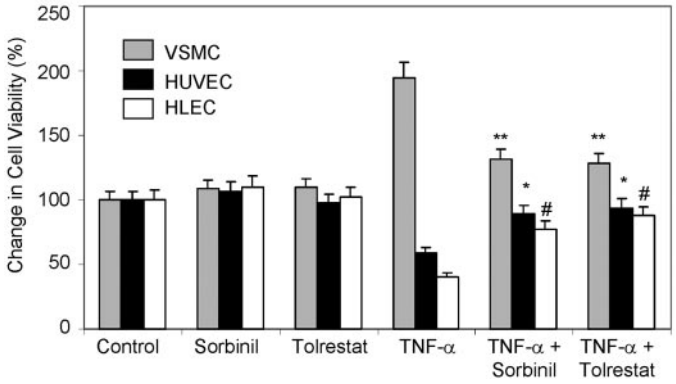

FIG. 7. Inhibition of AR attenuates proliferation of VSMC and apoptosis of human umbilical vein endothelial cells (HUVEC) and human lens epithelial cells (HLEC). Growth-arrested cells were pretreated with or without AR inhibitors, sorbinil and tolrestat (10 μM) for 24 h followed by stimulation with TNFα (2 nM) for an additional 24 h. Cell viability was determined by measuring the incorporation of [^{3}H]thymidine (10 μCi/ml), added 6 h before the end of the experiment. The extent of cell proliferation or apoptosis is expressed as percentage change in cell viability. **, $P < 0.001$; *, $P < 0.01$; and #, $P < 0.001$ in high glucose (HG) + AR inhibitor group compared with HG-treated group alone.

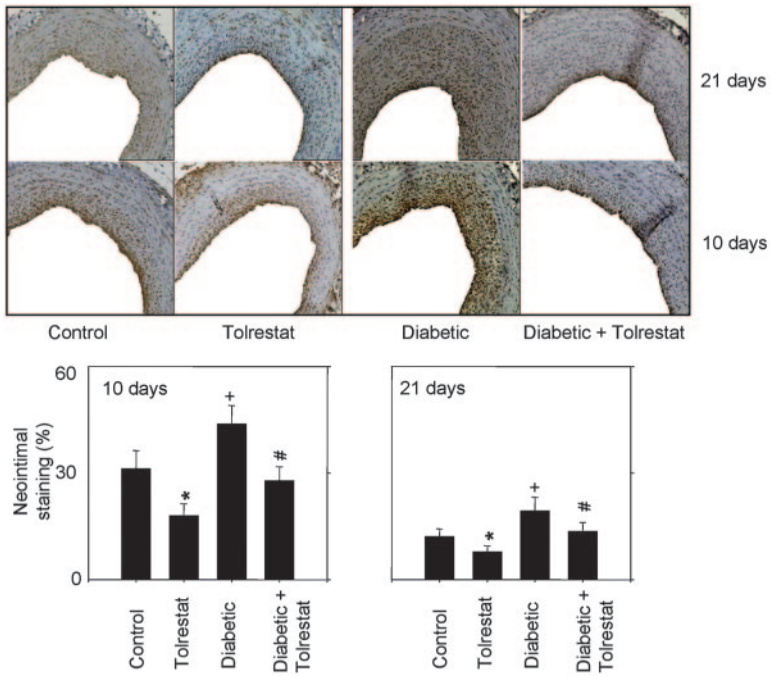

FIG. 8. AR inhibitor attenuates NF-κB activation in balloon-injured normal and diabetic rat arteries. Sections of balloon-injured arteries were obtained from nondiabetic and diabetic rats 10 and 21 d after balloon injury and were stained with anti-p65 antibodies. Immunoreactivity is evident as a *dark brown* stain, whereas the nonreactive areas display only the background color. The *bar graph* shows mean ± SEM values of the percent of neointima stained by the anti-p65 antibody. *, $P < 0.05$ in tolrestat *vs.* control; +, $P < 0.05$ in diabetic *vs.* control; and #, $P < 0.05$ diabetic + tolrestat *vs.* diabetic without tolrestat. [Reprinted with permission from K. V. Ramana *et al.*: *Diabetes* 53:2910–2920, 2004 (101). © 2004 American Diabetes Association.]

perplasia was associated with increased nuclear factor-κ-binding protein (NF-κB) activation (128). This was significantly prevented by sorbinil, suggesting that AR is required for signaling pathways responsible for NF-κB activation and for the proliferation of smooth muscle cells in vascular lesions. Together, these observations suggest that AR is essential for smooth muscle cell growth induced by several mitogenic pathways and that inhibition of AR interrupts their growth in culture and *in vivo* (53, 57, 128).

Although the mechanism by which AR regulates growth remains unclear, several of the key signaling pathways related to cell growth are sensitive to AR inhibition. Significantly, our studies reveal that inhibition of AR prevents activation of the transcription factor NF-κB stimulated with either TNFα (Fig. 9), FGF, angiotensin II, or high glucose (101, 128). Inhibition of AR, however, does not seem to directly interfere with the DNA binding of NF-κB, but prevents NF-κB activation by extinguishing signaling events upstream to dissociation of NF-κB from IκB and the nuclear translocation of NF-κB. This appears to be due to the inhibition of phosphorylation and degradation of IκBα in cells treated with AR inhibitors. Significantly, inhibition of AR does not prevent NF-κB when the cells are stimulated by phorbol esters, indicating that inhibition of AR does not interfere with the IκB-NF-κB signaling pathway but prevents signaling events upstream to PKC (128).

Similar to the effects observed with TNFα, inhibition or ablation of AR also interrupts the activation of the PKC-NF-κB by high glucose (101). The sensitivity of both high glucose- and TNFα-mediated cell growth to AR inhibitors suggests that both stimuli might activate overlapping pathways. Indeed, previous studies have shown that treatment with anti-TNFα antibodies prevents accelerated restenosis in diabetic vessels (129), and in our laboratory treatment with anti-TNFα antibodies was effective in preventing high glucose-induced smooth muscle cell growth in culture (K. V. Ramana, R. Tammali, A. Bhatnagar, and S. K. Srivastava, unpublished observations). Hence, it appears that the mitogenic effects of high glucose may be mediated, in part, by stimulation of cytokine release and autocrine activation of

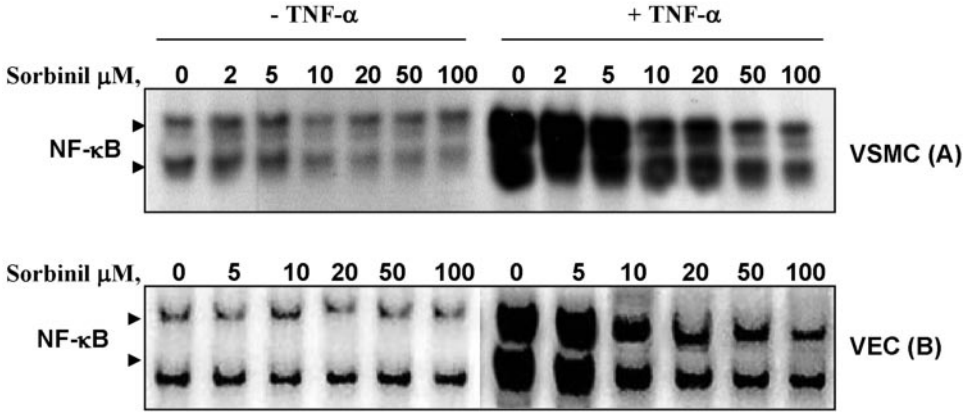

FIG. 9. Inhibition of AR attenuates TNFα-induced activation of NF-κB in VSMC and human umbilical vein endothelial cells (HUVEC). Quiescent VSMC (A) and HUVEC (B) were preincubated without or with the indicated concentrations of sorbinil for 24 h and then stimulated with 0.1 nM TNFα for 1 h. The nuclear extracts were prepared, and NF-κB activity was measured by EMSA. [A, Reproduced with permission from K. V. Ramana *et al.*: *J Biol Chem* 277:32063–32070, 2002 (128); and B, reproduced with permission from K. V. Ramana *et al.*: *FASEB J* 18:1209–1218, 2004 (134).]

the cell growth pathways. How glucose could induce the release of cytokines is unclear. However, the recent observation that the release of TNFα is regulated by PKC (130–132) suggests that activation of PKC by high glucose could trigger TNFα release and stimulate growth. Because inhibition/ablation of AR prevents both PKC activation (Fig. 10) and TNFα signaling, it could interrupt the initial trigger events as well as subsequent autocrine stimulation of mitogenic signaling in cells exposed to high glucose (101, 133).

Studies delineating the role of AR in intracellular signaling suggest an alternative paradigm for understanding the contribution of this enzyme to the development of diabetic complications and the efficacy of AR inhibitors against secondary diabetic complications, despite the detoxification role of the enzyme. Hence, inhibition of inflammation could represent one mechanism by which beneficial effects could be derived from inhibiting AR. Our studies show that key steps in the inflammatory process, such as NF-κB activation and the increase in the expression of adhesion molecules ICAM-1 and VCAM-1 and monocyte adhesion, could be prevented by inhibiting AR (Fig. 11) (134). Moreover, inhibition of AR also prevents the cytotoxic effects of TNFα on endothelial cells.

Our studies have also shown that inhibition of AR by sorbinil or tolrestat prevents TNFα-induced increase in Bax and Bad and the down-regulation of Bcl-2 (135, 136). Inhibition of AR also abrogates activator protein 1 DNA binding activity and prevents the activation of caspase-3, c-Jun N-terminal kinase, and p38 MAPK in cells stimulated by TNFα, suggesting that AR could be a critical regulator of TNFα-induced apoptotic signaling in endothelial cells (136). Given the key role of the endothelium in regulating atherogenesis and restenosis, the salutary effects of AR inhibitors on these cells could further contribute to the ability of these drugs to limit inflammation and vascular adhesion. Although the overall effects of inhibiting AR during atherosclerotic lesion formation remain to be studied, they are likely to be complex. Given that inhibition of AR could prevent inflammatory changes as well as increase the accumulation of lipid peroxidation products, inhibition of AR could yield highly context-dependent results, and the benefits of inhibiting AR may be specific to the extent of lesion progression and hyperglycemia and/or may require concurrent administration of antioxidants. Such duality and complexity of effects is, however, expected. AR regulates oxidative and inflammatory responses, both of which could be beneficial or harmful depending upon the context and the extent to which they are stimulated and the rate at which they are resolved. Regardless, much work remains to be done to further our understanding of the enigmatic role of AR and its contribution of antioxidant defenses as well as the development of secondary diabetic complications.

V. AR Inhibitors and the Treatment of Secondary Diabetic Complications

Despite the initial promise, outcomes of clinical trials with AR inhibitors have been disappointing. Most studies reveal only modest improvement with multiple side effects. However, despite such negative data, it would be unfortunate if anti-AR therapy were to be relegated to a historical footnote. Clinical trials with AR inhibitors were designed and conducted with only limited information about the enzyme and with little or no understanding of its physiological role in processes other than the reduction of glucose. Moreover, most clinical trials were designed to examine only a limited set of physiological and pathological end points and were mostly focused on diabetic neuropathy. However, subsequent to initial clinical trials, much has been learned about the enzyme and its role in glucose metabolism, detoxification, inflammation, and growth that will be critical in redesigning clinical trials with a larger number of pathological indices and end points.

Given the extensive data showing that by catalyzing the reduction of lipid-derived aldehydes and their glutathione conjugates (62, 66, 67), AR protects against oxidative stress (101, 128), it may be beneficial to try a combination therapy with AR inhibitor and antioxidants. Because long-term diabetes is associated with increased oxidative stress, the beneficial effects of AR inhibitors may be diminished by a concurrent accumulation of lipid peroxidation products. Therefore, to prevent this, it may be necessary to concurrently administer antioxidants that prevent lipid peroxidation or enhance the expression of antioxidant enzymes that can compensate for the lack of AR. Treatment with antioxidants may also keep the enzyme in the reduced form and

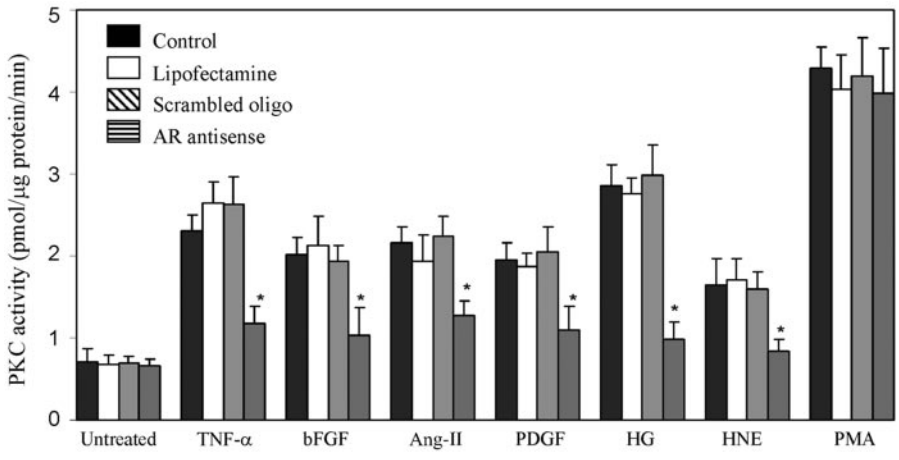

FIG. 10. Ablation of AR attenuates cytokines, growth factors, high glucose, HNE but not phorbol 12-myristate 13-acetate (PMA)-induced activation of PKC in VSMC. The VSMC were transiently transfected with AR antisense or scrambled control oligonucleotides. Subsequently, the cells were stimulated with TNFα (0.1 nM), basic FGF (bFGF) (5 ng/ml), platelet-derived growth factor (PDGF)-AB (5 ng/ml), angiotensin II (Ang-II; 2 μM), HNE (1 μM), high glucose (HG, 50 mM), or PMA (10 nM) for 4 h. The membrane-bound PKC activity was determined by using the Promega Signa TECT PKC assay system (Promega Corp., Madison, WI). *Bars* represent mean ± SE (n = 4). *, $P < 0.001$ in AR antisense group compared with control group. [A portion of the data was reproduced with permission from K. V. Ramana *et al.*: *J Biol Chem* 277:32063–32070, 2002 (128).]

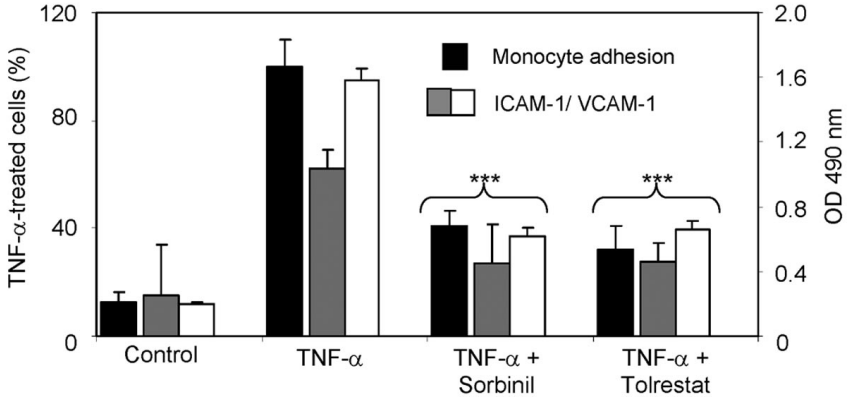

FIG. 11. Inhibition of AR attenuates TNFα-induced monocyte adhesion and cell surface expression of intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1 in human umbilical vein endothelial cells (HUVEC). Quiescent HUVEC were preincubated without or with 10 μM sorbinil/tolrestat for 24 h, and then stimulated with 2 nM TNFα for 24 h. After 24 h of incubation, THP-1 (monocytes) cells were added, and the incubation continued for another 12 h. Cell adhesion assays were performed using light microscope. To determine cell surface expression of adhesion molecules after stimulation with TNFα, the cells were fixed in 4% formaldehyde, and the expression of ICAM-1 and VCAM-1 was measured by ELISA using anti-VCAM-1 and anti-ICAM-1 antibodies, respectively. *Bars* represent mean ± SE (n = 4). ***, $P < 0.001$ in TNF-α + AR inhibitors-treated group compared with TNFα-treated group. [Reproduced with permission from K. V. Ramana *et al.*: *FASEB J* 18:1209–1218, 2004 (134).]

thus prevent drug resistance that develops due to AR modification under conditions of chronic hyperglycemia. Additionally, treatment with AR inhibitors could be restricted to a specific stage of disease development. Thus, for instance, inhibition of AR may be more beneficial during early stages of diabetes, when oxidative stress is low, than during later stages of the disease when, due to increased oxidative stress and oxidative modification of the enzyme, it may be difficult to inhibit AR or to accrue significant benefits from anti-AR interventions without further increasing oxidative stress.

Although changes in cellular metabolism and tissue injury have been monitored in both animal studies and clinical trials, changes in inflammation and inflammatory markers have not been carefully examined. In view of recent studies showing that inhibition of AR prevents multiple inflammatory pathways (Refs. 101, 128, and 134; Fig. 12), it may be necessary to examine how AR inhibitors affect systemic and local inflammation during diabetes. This appears to be particularly critical because chronic inflammation has emerged to be one of the critical features of diabetic complications, particularly cardiovascular disease. Moreover, based on data showing that inhibition of AR prevents restenosis, vascular smooth muscle growth, and endothelial cell apoptosis (101, 134–136), it may be possible to design animal and clinical studies to test the efficacy of anti-AR therapy against micro- and macrovascular complications of diabetes, which appear to be the most serious and prevalent outcomes of prolonged diabetes. Because protection against vascular changes was accompanied by an inhibition of cell signaling involved in inflammation, it appears likely that AR inhibitors may be

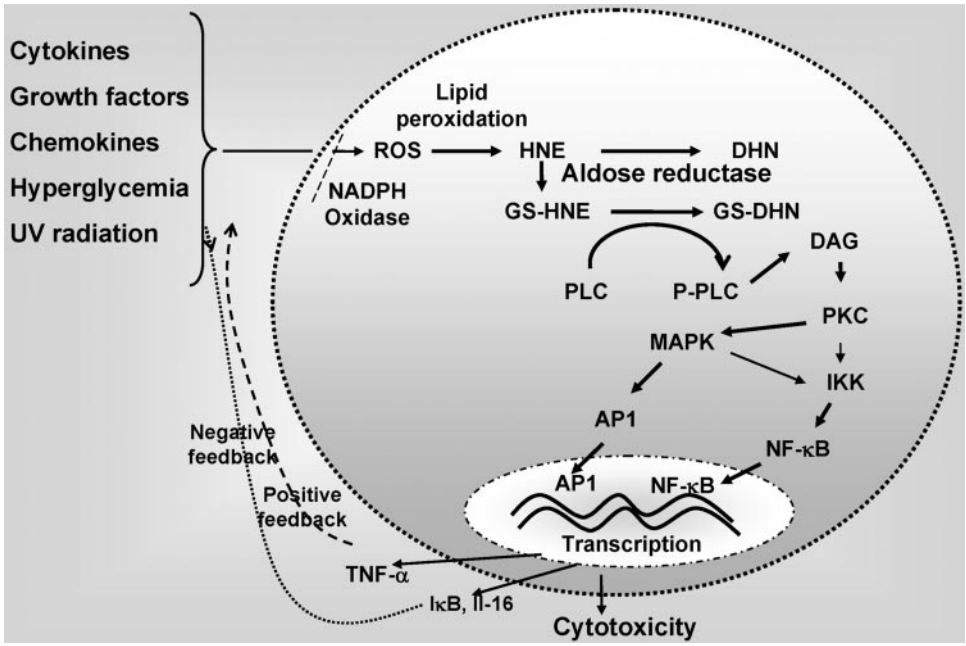

FIG. 12. Schematic representation of AR involvement in cell signaling. Cytokines, growth factors, and hyperglycemia generate ROS and cause peroxidation of lipids resulting in the generation of toxic lipid aldehydes such as HNE. AR efficiently reduces HNE and its conjugate with glutathione (GSH) to 1–4-dihydroxy-2-nonene (DHN) and GS-DHN, respectively. The reduced products of aldehydes may be involved in the cytotoxic signaling leading to cell death or growth via activation of phospholipase C (PLC)/PKC/NF-κB pathway. Paracrine effects of cytokines such as TNFα amplify hyperglycemia and other signals as shown in the pathway.

effective against diseases other than diabetes, particularly those that are associated with high levels of cytokine generation and inflammation such as rheumatoid arthritis and sepsis. Acute intervention with AR inhibitors during sepsis appears to be particularly attractive because short-term treatment will promote recovery by inhibiting both cytokine signaling and production, without the risk of long-term treatment with AR inhibitors that may chronically increase oxidative stress.

Finally, to derive maximal benefits from AR inhibition, it is imperative to have specific and selective inhibitors. Although the currently available AR inhibitors bind to the enzyme with high affinity, they also display high levels of nonspecific toxicity. Moreover, in the absence of detailed pharmacokinetic studies, it is unclear whether the dose of AR inhibitors used for clinical studies was effective in inhibiting AR. Hence, further studies are warranted to carefully determine the extent of enzyme inhibition in human tissues for a given dose of AR inhibitor and whether their efficacy persists in diabetic tissues. More importantly, however, it appears that it may be necessary to design more specific inhibitors of AR that could selectively inhibit the ability of the enzyme to catalyze the reduction of glutathione conjugates and glucose, without inhibiting aldehyde detoxification. Our structure-activity studies (66, 67) with free and glutathione-conjugated aldehydes suggest that there are distinct glutathione- and aldehyde-binding domains on the enzyme, and selective modification of the enzyme active site could prevent recognition and reduction of glutathione conjugates without affecting aldehyde reduction. These results suggest the interesting possibility that the signaling and detoxification roles of AR could be regulated independently of each other and that more selective inhibitors could be designed to selectively prevent cell injury without compromising antioxidant defense.

VI. Conclusion

Due to its ability to reduce a wide variety of aldehydes, ranging from membrane phospholipids to glucose, AR plays a complex role in cellular metabolism and signaling. Although identified initially as a glucose-reducing enzyme, the enzyme is now believed to be an important component of antioxidant defense involved in the removal and detoxification of reactive aldehydes generated by lipid peroxidation. Inhibition of AR has been shown to prevent the development of diabetic complications in animal models; however, a critical evaluation of the clinical efficacy of AR inhibitors awaits a clearer understanding of the role of AR in regulating inflammation and cell growth. More selective and effective inhibitors are needed to specifically inhibit the cytotoxic role of AR in cell signaling without affecting its detoxification role. Such inhibitors are likely to be more effective in treating secondary diabetic complications by preventing inflammation due to chronic hyperglycemia.

Acknowledgments

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This work was supported, in part, by NIH Grants DK36118, EY01677, HL55477, and HL59378.

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