

Bioinformatics analysis of diabetic retinopathy using functional protein sequences

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Abstract

Diabetic retinopathy is the leading cause of blindness among patients with diabetes mellitus. We evaluated the role of several proteins that are likely to be involved in diabetic retinopathy by employing multiple sequence alignment using ClustalW tool and constructed a phylogram tree using functional protein sequences extracted from NCBI. Phylogram was constructed using Neighbor-Joining Algorithm in bioinformatics approach. It was observed that aldose reductase and nitric oxide synthase are closely associated with diabetic retinopathy. It is likely that vascular endothelial growth factor, pro-inflammatory cytokines, advanced glycation end products, and adhesion molecules that also play a role in diabetic retinopathy may do so by modulating the activities of aldose reductase and nitric oxide synthase. These results imply that methods designed to normalize aldose reductase and nitric oxide synthase activities could be of significant benefit in the prevention and treatment of diabetic retinopathy.

Key words: Diabetic retinopathy, nitric oxide, vascular endothelial growth factor, aldose reductase, advanced glycation end products, cytokines, adhesion molecules, diabetes mellitus

Introduction

Diabetic retinopathy, a microvascular complication of hyperglycemia, can result in blindness. Multiple interlinked biochemical mechanisms have been postulated to be involved in diabetic retinopathy. The mechanisms mainly include: increased aldose reductase activity that results in enhanced flux of glucose through the polyol pathway, formation of advanced glycation end product (AGEs), activation of protein kinase C (PKC), enhanced formation of reactive oxygen species (ROS), increased production of vascular endothelial growth factor (VEGF) and placental growth factor (PIGF), and reduced generation of endothelial nitric oxide (eNO) (1-8). In addition, studies in animal and cell culture models revealed that impaired growth factor support, enhanced oxidative/nitrosative stress, and its downstream effectors such as mitogen-activated protein kinase activation, inflammatory response, endothelin-1 overexpression and impaired Ca^{2+} signaling also play an important role in the pathogenesis of diabetic retinopathy. Evidence for important role of the downstream effector of free radical and oxidant-induced DNA injury, poly(ADP-ribose) polymerase activation, is emerging (9).

Several animal studies revealed that there is strong evidence that aldose reductase, the first and rate-limiting enzyme of the polyol pathway that converts glucose to fructose, plays a key role in the pathogenesis of microvascular complications including diabetic retinopathy (10-15). Aldose reductase plays an important role in the pathogenesis of

diabetic retinopathy by inducing retinal lesions including blood retinal barrier break down, loss of pericytes, neuroretinal apoptosis and glial reactivation and neovascularization-events that are associated with diabetic retinopathy (15). Animal studies revealed that administration of aldose reductase inhibitors to diabetic rats prevented basement membrane thickening, pericyte loss, and development of microaneurysms in the retinal capillaries (15). These results emphasize the importance of aldose reductase in the pathogenesis of diabetic retinopathy. However, clinical trials of the aldose reductase inhibitors were disappointing, suggesting that changes in the concentrations and activities of other proteins and enzymes also play a significant role in the pathogenesis of diabetic retinopathy and/or cooperate with aldose reductase to induce the development of diabetic retinopathy. In order to evaluate the role of other such factors in the development of diabetic retinopathy, the present study was performed to identify key protein(s) contributing to diabetic retinopathy using bioinformatics tools. We sought to verify the role of various molecules believed to be involved in the pathogenesis of diabetic retinopathy using bioinformatics approach by employing multiple sequence alignment using ClustalW tool and constructed a phylogram tree employing functional protein sequences extracted from NCBI. Phylogram was constructed using Neighbor-Joining Algorithm in this bioinformatics approach.

Material and Methods

We collected 28 known genes that are believed to be involved in the pathogenesis of diabetic retinopathy. The functional protein sequences in FASTA for these genes are collected from NCBI (National Center for Biotechnology information,

<http://www.ncbi.nlm.nih.gov/>). These sequences are given to ClustalW (<http://www.ebi.ac.uk/clustalw/>) for the Multiple Sequences Alignment (it calculates the best match for the selected sequences, and lines them up so that the identities, similarities and differences can be seen). Based on these results, the Scores Table and Phylogeny Tree are derived. The phylogeny tree shows the distance between the protein sequences. The proteins with minimum distance are aldose reductase and nitric oxide synthase.

Results

Analysis of various proteins involved in the pathogenesis of diabetic retinopathy using Multiple Sequences Alignment and construction of the scores table (see table 1) revealed that 28 proteins are closely associated with this disease process. Construction of the phylogeny tree using (see Figure 1) this data revealed that of all the proteins studied aldose reductase and nitric oxide synthase are the two proteins with minimum distance indicating a dominant role for them in diabetic retinopathy. These results suggest that aldose reductase not only interacts with nitric oxide synthase but also with several other proteins such that various pathogenic events seen in diabetic retinopathy are initiated and allowed to progress.

Discussion

Aldose reductase is the first and rate-limiting enzyme of the polyol pathway. Under normoglycemic conditions, aldose reductase plays a minor role in glucose metabolism. In contrast, persistent hyperglycemia due to uncontrolled diabetes leads to a significant increase in aldose reductase activity. This increase in polyol pathway due to

hyperglycemia could lead to complications seen in diabetes such as retinopathy, neuropathy and nephropathy. An increase in aldose reductase activity results in sorbitol accumulation that produces osmotic stress leading to loss of cellular integrity and function (10). This is supported by the observation that increase in the prevalence of diabetic retinopathy is positively associated with an increase in enhanced erythrocyte aldose reductase levels in patients with type 2 diabetes (16). Several reports suggested that aldose reductase gene polymorphism is associated with development of diabetic retinopathy (12, 17-19). Although treatment with aldose reductase 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 revealed that glucose might be an incidental substrate of aldose reductase, which appears to be more adept in catalyzing the reduction of a wide range of aldehydes generated from lipid peroxidation. Inhibition of aldose reductase enzyme has been shown to increase inflammation-induced vascular oxidative stress and prevent myocardial protection associated with the late phase of ischemic preconditioning. These studies indicate that aldose reductase enzyme has potent antioxidant actions. Furthermore, aldose reductase is a critical component of intracellular signaling, and its inhibition prevents high glucose-, cytokine-, or growth factor-induced activation of PKC and nuclear factor-kappa-binding (NF- κ B) protein. Thus, it is anticipated that aldose reductase inhibitors prevent vascular smooth muscle cell growth and endothelial cell apoptosis and inflammation and thus, aid in the prevention or arrest of retinopathy in type 2 diabetes mellitus. It is likely that the antioxidant and signaling roles of aldose reductase are interlinked and that aldose reductase regulates PKC and NF- κ B via redox-sensitive mechanisms (20). These results emphasize the need for

development of drugs that selectively inhibit aldose reductase-mediated glucose metabolism and signaling, without affecting aldehyde detoxification in the prevention of inflammation associated with the development of diabetic retinopathy.

When the relationship between increased aldose reductase activity and abnormal endothelium-dependent relaxation was examined in experimental animals with alloxan-induced diabetes, it was noted that basal and acetylcholine-stimulated levels of cyclic GMP and the relaxations in response to an endothelium-independent vasodilator, sodium nitroprusside, were not significantly different between diabetic and normal rabbits, indicating that nitric oxide release and action on the vascular smooth muscle were unchanged. The release of thromboxane A₂ from diabetic vessels was found to be increased, whereas aldose reductase inhibitor, zopolrestat, normalized the elevated red blood cell sorbitol levels in diabetic rabbits, and restored the abnormal acetylcholine- and adenosine diphosphate-induced relaxations of the aorta. On the other hand, aldose reductase inhibitor had no effect on the levels of cyclic GMP or on the increased release of thromboxane A₂ in diabetic aorta. These findings suggest that increased activity of the aldose reductase pathway in hyperglycemia is responsible for the abnormal endothelium-dependent relaxation in diabetic blood vessels (21). In this context, it is interesting to note that aldose reductase and nitric oxide synthase (NOS) share NADPH as an obligate cofactor, suggesting that enhanced glucose flux by aldose reductase inhibited NO production by blunting NOS activity (22). However, aldose reductase inhibitors prevented the inhibition of NO production, implying that aldose reductase inhibitors decrease glucose-mediated inhibition of NO production and thus, ameliorate endothelial function associated with diabetes.

Human umbilical vein endothelial cells (HUVECs) cultured in the presence of a high concentration of glucose (27.8 mM for 48 h) increased neutrophil-endothelial cell adhesion and surface expression of intercellular adhesion molecule-1 (ICAM-1), P-selectin, and E-selectin on endothelial cells, which was significantly inhibited by epalrestat, an aldose reductase inhibitor, while NO synthase (NOS) inhibitors reduced the inhibitory effects of this compound. In contrast, phorbol 12-myristate 13-acetate (PMA), a PKC activator, showed similar effects as high glucose, and these effects were also inhibited by epalrestat, suggesting that aldose reductase inhibitors inhibit high glucose-mediated neutrophil-endothelial cell adhesion and expression of endothelial adhesion molecules not only through inhibition of a PKC-dependent pathway, but also through increased endothelial NO production (23). These results are supported by the observation that exposure of cultured retinal endothelial cells to high glucose levels and osmotic stress similar to those in diabetic patients increased the formation of nitrotyrosine by increasing NOS activity and causing superoxide formation due to eNOS uncoupling and aldose reductase activation (24). Thus, there is a close interaction between aldose reductase activity and eNO generation and the development of diabetic retinopathy. These results also suggest that increase in eNO generation is a compensatory phenomena in response to enhanced oxidative stress induced by hyperglycemia since inhibiting NOS or aldose reductase, scavenging superoxide or peroxynitrite, or supplementing the NOS substrate L-arginine or cofactor tetrahydrobiopterin blocked the formation of reactive oxygen species and prevented protein tyrosine nitration (24). This is supported by the observation that increased flux of glucose through the polyol pathway is involved in the pathophysiology of secondary diabetic complications, and the first step of this pathway,

which generates sorbitol from glucose, is catalyzed by aldose reductase. In vitro, the binding of substrates and inhibitors to aldose reductase is highly sensitive to the oxidation state of the enzyme due to the presence of a hyper-reactive cysteine residue (Cys-298) at the active site of the enzyme that can be readily modified by thiol-modifying reagents, nitric oxide (NO) donors and nitrosothiols. Studies revealed that exposure of erythrocytes to NO donors inhibited aldose reductase activity and aldose reductase-mediated accumulation of sorbitol, suggesting that NO regulates the cellular activity of aldose reductase and, in turn, the flux of glucose via the polyol pathway. This inhibition of aldose reductase by exogenous or endogenous NO is related to reversible S-glutathiolation of the aldose reductase protein (25). Since, hyperglycemia is associated with a decrease in NO generation (26, 27), the loss of NO-mediated repression of aldose reductase is a significant factor in the activation of the polyol pathway and the development of diabetic retinopathy and other diabetic complications. These results also suggest that NO is a physiological regulator of aldose reductase. Furthermore, it was reported that NOS inhibitor, N(G)-nitro-L-arginine methyl ester (L-NAME), increased sorbitol accumulation, whereas treatment with L-arginine (a precursor of NO) or nitroglycerine patches prevented sorbitol accumulation. When incubated ex vivo with high glucose, sorbitol accumulation was increased by L-NAME and prevented by L-arginine in wild type, but not eNOS-deficient, mice. Exposure to NO donors also inhibited aldose reductase and prevented sorbitol accumulation in rat aortic vascular smooth muscle cells (VSMC) in culture (25, 28). These observations suggest that NO regulates the vascular synthesis of polyols by S-thiolating aldose reductase and, therefore,

increasing NO synthesis or bioavailability may be useful in preventing diabetes-induced changes in the polyol pathway and secondary complications due to diabetes mellitus.

Previous studies also suggested a link between aldose reductase and VEGF. It was noted that enhanced aldose reductase activity triggers retinal oxidative stress and VEGF protein expression, events that could be prevented by aldose reductase inhibitor (29).

Impaired eNOS activity seen in diabetes causes vasospasm that results in hypoxia. The resultant hypoxia augments VEGF synthesis that could induce increase in vascular permeability. Impaired basal NO production facilitates leukocyte adhesion to the endothelium, which would result in the breakdown of the blood retinal barrier (BRB) leading to capillary non-perfusion. Alternatively, impaired eNOS activity may directly increase microvascular permeability (14).

Based on the results of the previous studies and the Phylogram constructed using Neighbor-Joining Algorithm using bioinformatics approach of the present study, it is suggested that hyperglycemia induces increase in the activity of the enzyme aldose reductase that, in turn, triggers a series of events leading to enhanced expression of iNOS, VEGF, PIGF (placental growth factor) and free radicals and activation of PKC. These molecular cause endothelial cell migration and replication (early steps in angiogenesis), enhance retinal vascular permeability that ultimately lead to the onset of diabetic retinopathy. AGE-RAGE interaction also elicits angiogenesis through the transcriptional activation of the VEGF gene vial NF- κ B (30) that can be prevented by aldose reductase inhibitors. This suggests that interaction between aldose reductase and VEGF is crucial in the pathogenesis of diabetic retinopathy. VEGF, in turn, works in concert with angiopoietins, a set of growth factors that modulate physiological angiogenesis and

pathological neovascularization, particularly in association with VEGF (31).

Furthermore, adhesion of leukocytes to the retinal vasculature is one of the earliest events in experimental diabetes and results in blood retinal breakdown, endothelial cell damage and capillary non-reperfusion. ICAM-I and other adhesion molecules are up-regulated during diabetic retinopathy and VEGF drive the up-regulation of retinal ICAM-I, mostly via NO and NF- κ B dependent pathways.

Conclusion

It is evident from the preceding discussion that diabetic retinopathy is a complex process in which several cytokines, growth factors, and free radicals play a significant role. In general, it is likely that hyperglycemia causes an increase in the activity of the enzyme aldose reductase that, in turn, triggers a series of events leading to enhanced expression of iNOS, VEGF, PIGF and free radicals. However, clinical trials of the aldose reductase inhibitors were disappointing, whereas VEGF antagonists showed limited beneficial actions suggesting that changes in the concentrations and activities of other proteins and enzymes such as endothelial nitric oxide synthase and various growth factors also play a significant role in the pathogenesis of diabetic retinopathy. Consistent with this observation, the present bioinformatics study suggests a close association exists between aldose reductase, VEGF, NOS, PIGF, AGE-RAGE, angiopoietins, and cytokines in the pathogenesis of diabetic retinopathy (Figure 2) and hence, a multi-pronged approach is needed to tackle this condition. Hence, development of drugs that inhibit aldose reductase and simultaneously enhance eNOS activity will be useful in the

prevention and treatment of diabetic retinopathy and other complications of diabetes mellitus.

Competing Interests:

The author(s) declared that they have no competing interests.

Authors' Contributions:

AAR, THOTA participated in the design of the study, interpretation of the results and prepared manuscript in bioinformatics aspects. GRS, UND, AA participated in the design of study, interpretation of the results and prepared manuscript. SBC, SRC, SPA, TP, DC and SK participated in the design of the study, performed bioinformatics aspects and participated in the preparation of manuscript. All authors read and approved the final manuscript.

PHYLOGRAM

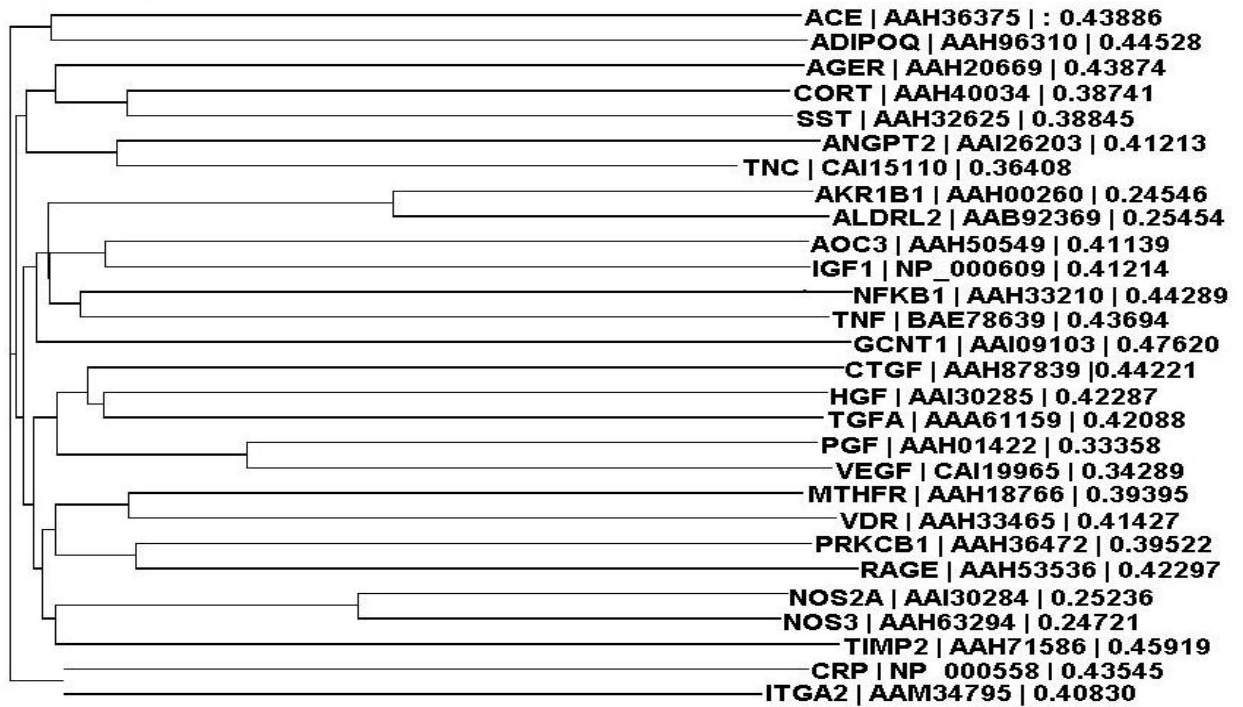


Figure 1. Phylogram constructed using Neighbor-Joining Algorithm in bioinformatics approach.

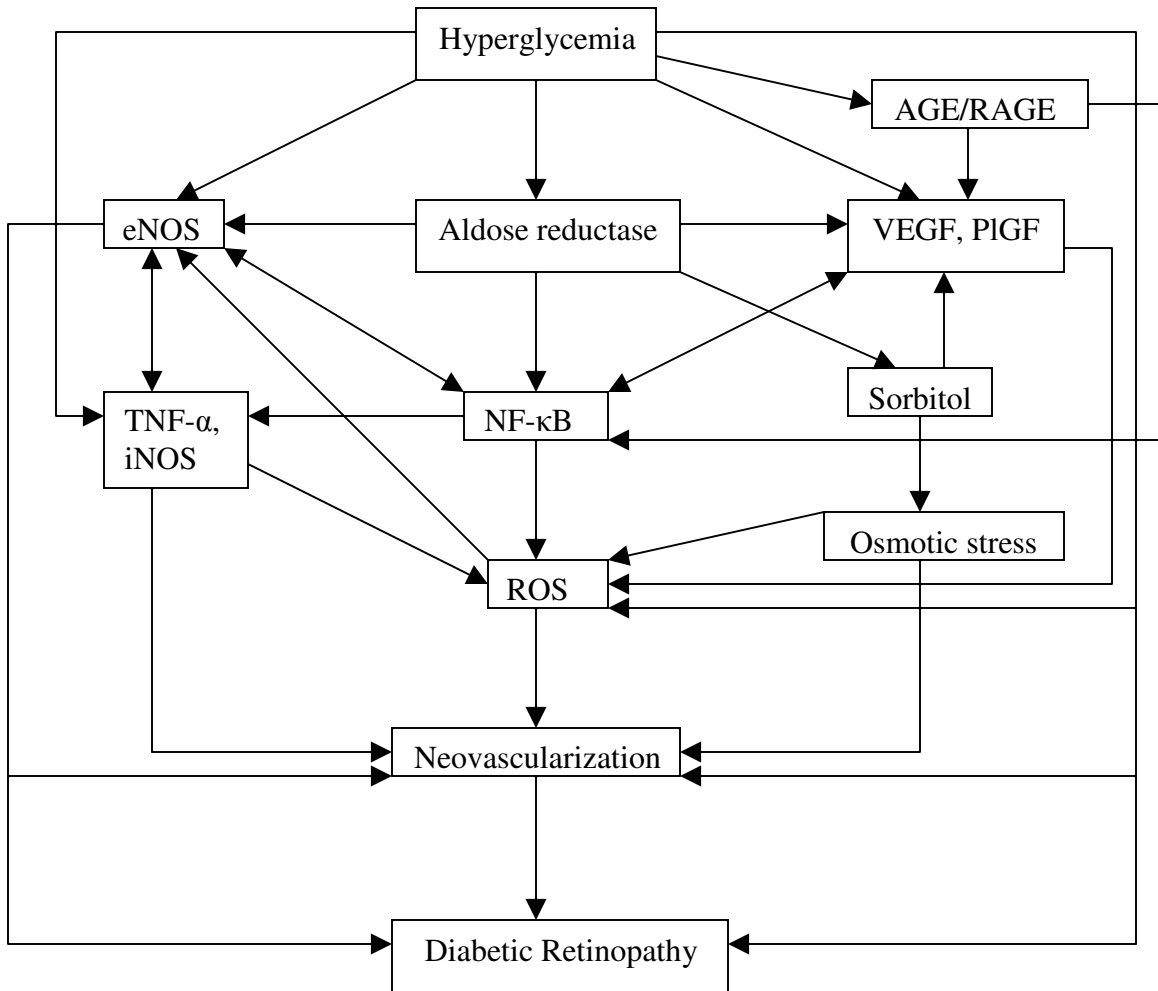


Figure 2. Scheme showing the role of various factors involved in the pathogenesis of diabetic retinopathy and their interaction(s).

S No.	GENE SYMBOL	PROTEIN ID	TISSUE TYPE	LENGTH	REFERENCES
1	ACE	AAH36375	Testis	739 aa	32, 33
2	ADIPOQ	AAH96310	PCR rescued clones"	244 aa	34
3	AGER	AAH20669	lung	404 aa	35, 36, 37
4	AKR1B1	AAH00260	Eye, retinoblastoma	316 aa	14, 29
5	ALDRL2	AAB92369	No tissue type	325 aa	14-17
6	ANGPT2	AAI26203	Colon, PCR rescued clones	496 aa	38
7	AOC3	AAH50549	Peripheral Nervous System, sympathetic trunk	763 aa	39
8	CORT	AAH40034	Brain, adult, 6 pooled whole brains	122 aa	40
9	CRP	NP_000558	LIVER	224 aa	41
10	CTGF	AAH87839	Peripheral Nervous System, dorsal root ganglion	349 aa	42-45
11	GCNT1	AAI09103	PCR rescued clones	428 aa	46
12	HGF	AAI30285	Brain, cerebellum, PCR rescued clones	728 aa	47, 48
13	IGF1	NP_000609	-No Tissue Type-	153 aa	49, 50
14	ITGA2	AAM34795	-No Tissue Type-	1181 aa	51, 52
15	MTHFR	AAH18766	Eye, normal, pigmented retinal epithelium	73 aa	53
16	NFKB1	AAH33210	Muscle, rhabdomyosarcoma	550 aa	54, 55
17	NOS2A	AAI30284	Pooled, cerebellum, kidney, placenta, testis, lung, colon, liver, heart, thyroid, bladder, uterus, PCR rescued clones	1153 aa	56, 57
18	NOS3	AAH63294	Placenta, normal	1203 aa	58, 59
19	PGF	AAH01422	Placenta, choriocarcinoma	170 aa	46, 60
20	PRKCB1	AAH36472	Brain, hippocampus	673 aa	61
21	RAGE	AAH53536	Brain, Lung, Testis, adult, pooled whole	231 aa	62
22	SST	AAH32625	Brain, fetal, whole pooled	116 aa	63
23	TGFA	AAA61159	renal carcinoma	160 aa	44
24	TIMP2	AAH71586	Placenta, pre-eclamptic	220 aa	64, 65
25	TNC	CAI15110	-No Tissue Type-	2201 aa	66
26	TNF	BAE78639	Peripheral blood leukocyte	233 aa	67
27	VDR	AAH33465	Brain, Lung, Testis, adult, pooled whole	473 aa	68
28	VEGF	CAI19965	-No Tissue Type-	191 aa	69, 70

Table 1. Genes (28 in number) that are believed to be involved in the pathogenesis of diabetic retinopathy with their respective gene symbol and protein ID that have been analysed in the present study.

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