Angiogenic response of advanced glycation end products (AGEs) involves PPAR γ

Manju S Devi and Perumana R Sudhakaran*

Department of Biochemistry, University of Kerala, Kariavattom, Thiruvananthapuram 695 581, Kerala, India

Received 17 June 2011; revised 28 November 2011

Diabetes is associated with increased formation of advanced glycation end products (AGEs), which have been implicated in micro and macrovascular complications of diabetes. Our earlier reports showed proangiogenic effect of AGE-bovine serum albumin (BSA). In order to understand the mechanism of AGE-mediated angiogenesis, the possibility of involvement of peroxisome proliferator activated receptor (PPAR) γ, a ligand activated transcription factor was examined. The angiogenic effect was studied in chick chorio allantoic membrane (CAM) and by analyzing angiogenic markers in human umbilical vein endothelial cells (HUVECs) in culture. The involvement of PPAR γ was investigated using synthetic PPAR γ agonist GW 1929 and antagonist GW 9662 and by RT-PCR. In CAM assay, PPAR γ antagonist GW 9662 reversed the AGE-induced effect on vascularity. In HUVECs in culture, GW 9662 reversed the effect of AGE-BSA and decreased the expression of CD 31, E-Selectin and VEGF. RT-PCR analysis showed that treatment with AGE-BSA caused upregulation of PPAR γ mRNA levels. The reversal of the effect of AGE on angiogenesis by treatment with PPAR γ antagonists and up-regulation of PPAR γ gene in HUVECs treated with AGE-BSA suggested the possible involvement of PPAR γ-dependent downstream pathway in mediating the angiogenic effect of AGE.

Keywords: Advanced glycation end products (AGEs), PPAR γ, Angiogenesis, Diabetes, VEGF

Several of the long-term complications of diabetes mellitus are characterized by vasculopathy associated with aberrant angiogenesis. Excessive angiogenesis plays a role in diabetic retinopathy and nephropathy, whereas suppression of angiogenesis contributes to impaired wound healing, impaired coronary collateral vessel development, embryonic vasculopathy in pregnancies complicated by maternal diabetes and transplant rejection in diabetic recipients.

Increased formation of advanced glycation end products (AGEs) is generally regarded as one of the mechanisms responsible for vascular damage in patients with the diabetes. In diabetic condition, non-enzymatic reaction occurs between glucose and protein/lipoprotein in arterial wall. Some of the early glycosylation products on long-lived proteins continue to undergo complex series of chemical rearrangements to form AGEs. AGE-protein adducts so formed are stable and virtually irreversible. AGEs contribute to diabetic complications through the formation of cross-links between key molecules in the basement membrane of the extracellular matrix, altering the cellular structure. AGEs formed on intracellular proteins alter the normal function of endothelial cells, monocytes and smooth muscle cells and alter cellular properties that are critical in vascular homeostasis.

AGEs are involved in aberrant angiogenic potential in diabetic condition. Recently, we have shown that AGE-bovine serum albumin (BSA) induced angiogenesis in different model systems. In human umbilical vein endothelial cells (HUVECs) in culture, treatment with AGE-BSA induced angiogenesis through the upregulation of VEGF. AGE exerts its cellular effect by binding to specific receptors, the most important one being the receptor for advanced glycation end product (RAGE). Other receptors, such as scavenger receptor, AGE R1 and Galectin-3 are also involved in mediating AGE effect. These modified
proteins are also taken up by macrophages and partially degraded to form reactive AGE peptides.

Peroxisome proliferator activated receptors (PPARs) are ligand-activated supergene family of nuclear transcription factors differentially expressed in most tissues. PPARs have recently received increased attention with regard to cardioprotective mechanisms by modulating such pathologic entities as hypertrophy, apoptosis and inflammation. PPARs regulate gene expression upon ligand binding that drives heterodimerization with the retinoid X receptor (RXR) and subsequent binding to specific response elements located in the promoter regions of target genes. Of the different isoforms of PPARs, PPARγ is expressed in all cell types including vascular smooth muscle cells (VSMC), endothelial cell (EC) and macrophages in the vasculature. It is involved in a number of pathways including cell-cycle regulation, and vascular function. Apart from being a potent modulator of the EC and VSMC function, PPARγ is known to be a key regulator of glucose homeostasis.

PPAR-γ ligands constitute important insulin sensitizers that have already been approved for the treatment of human metabolic disorders. Ligands of PPAR-γ include prostaglandin derivatives and insulin-sensitizing drugs, such as glitazones. Recent data have shown that PPAR-γ ligands act as potential modulators of angiogenesis both in vitro and in vivo. PPARγ is reported to have both anti-and proangiogenic effect. As PPARγ is a ligand activated transcription factor, in this study, we have examined whether effect of AGEs on angiogenesis involves PPARγ-dependent mechanisms. Results of experiments presented here using synthetic agonist GW 1929 and antagonist GW 9662 of PPARγ suggest that AGEs-mediated angiogenesis depends on PPARγ pathway.

Materials and Methods

Collagenase, MCDB 131-medium, antibiotic-antimycotic solution, o-phenylenediamine dihydrochloride, diethyl pyrocarbonate, diaminobenzidine, Tris, glycine, protease inhibitor cocktail, bovine serum albumin (BSA), protein A sepharose, monoclonal antibodies against VEGF, E-selectin, CD31 (PECAM1), horse radish peroxidase (HRP)-conjugated secondary antibody, GW 1929 and GW 9662 were purchased from M/s Sigma Aldrich Co, St. Louis, MO, USA. Nitrocellulose (NC) membrane was from BIORAD laboratories, Hercules, CA. Tissue culture plastic wares were purchased from NUNC A/S, Roskilde, Denmark. All other reagents used were of extra pure quality from Merck Ltd., Mumbai, India. Chick embryos were obtained from regional poultry farm, Trivandrum.

Isolation of human umbilical vein endothelial cells (HUVECs)

Endothelial cells were isolated by collagenase perfusion of the human umbilical vein as described before. The viability of isolated HUVECs was determined by trypan blue exclusion. Cells in MCDB131 medium were seeded in tissue culture plates and maintained in a Sanyo CO2 incubator at 37°C in 95% air/5% CO2 atmosphere.

Chick CAM assay

Chorio allantoic membrane (CAM) assay was performed as described earlier. Briefly, fertilised chick eggs (White Leghorn) were incubated for 8 days at 37°C and at a relative humidity of 80%. During this period, the eggs were positioned with pointed end down and rotated several times. After the incubation, the eggs were opened on the air sac side, shell was carefully removed with forceps and samples soaked in filter discs (4 mm diameter) were applied on to the CAM. The cavity was covered with parafilm and the eggs were incubated at 37°C at a relative humidity of 80% for further 4 days. At the end of incubation period, the CAMs were photographed. The level of haemoglobin (Hb) in the CAM was estimated using Drabkin’s reagent as a measure of vessel density. The CAM was homogenised in Drabkin’s reagent, centrifuged at 5,000 rpm for 15 min and the absorbance of clear supernatant was recorded at 546 nm. Hb was expressed as mg/g of CAM protein.

Preparation of AGE-BSA

BSA (1 mg/ml) was incubated in tris buffered saline (TBS 0.1 M Tris, 0.15 M NaCl, pH 7.4) containing 300 mM glucose and 0.01% NaN3 for six weeks at 37°C in an air incubator. For determination of AGEs formation, the fluorescence intensities of both glycated and non-glycated BSA solutions were recorded at excitation/emission wavelengths of 370/440 nm. About 600-fold increase in fluorescence intensity for AGE-BSA compared to untreated BSA was observed.

ELISA

Amount of VEGF, E-selectin and CD31 (PECAM1) were quantitated by ELISA using HRP conjugated secondary antibody. o-Phenylenediamine dihydrochloride was used as substrate. Color intensity at 495 nm was read in a multi-well microplate reader (Thermo Multiskan Spectrum).
Western blot

VEGF and E-selectin production was also determined by Western blot analysis. Proteins were separated in a 10% polyacrylamide gel and transferred to nitrocellulose membranes and probed using specific monoclonal antibodies (dilution 1: 500), followed by secondary anti-mouse IgG conjugated to HRP (dilution of 1: 2,000). The bands were detected by staining with diaminobenzidine and the relative intensity of bands was quantitated using BIORAD Quantity One version 4.5 software in a BioRad gel doc.

Relative quantitative reverse transcription-PCR (RT-PCR)

Total RNA from HUVECs maintained in culture was isolated using Perfect RNA Mini isolation kit (Sigma) according to manufacturer’s instruction. The nucleotide sequence of primer pairs used to determine the levels of human VEGF, and GAPDH (glyceraldehydephosphate dehydrogenase) mRNA were as follows: VEGF (105 bp) sense primer 5’ACGATCGATACGAAACCACG3’ and anti-sense primer 5’CTCTGCGAGAGTCTCCTCT3’; GAPDH (680 bp) sense primer 5’CGGAGTCT AACGGATTGCTGTA3’ and anti-sense primer 5’GCAGTCAGGTCCACCACT GAC-3’. Primer pairs for human PPARγ were as follows: forward primer: 5’-CATGTGACAATGATACGCC-3’ and reverse primer: 5’ GCCAAGGCCCTTATTTAAG-3’. The primer sequences were selected from NCBI nucleotide database and custom synthesized from Sigma Chemicals Ltd. RT-PCR was performed in an Eppendorf thermocycler as described before.

Statistical analysis

Results were expressed as mean with standard error of mean (SEM). The statistical significance was analyzed by Duncan’s One-way-Analysis-of-Variance (ANOVA) using SPSS 11.0 Software. A value of P<0.05 was considered significant.

Results

Effect of PPAR γ antagonist and agonist on AGE-BSA induced angiogenesis in chick CAM

The involvement of PPAR γ in AGE-mediated angiogenesis was examined in CAM model system. Consistent with the earlier data, treatment with AGE-BSA caused significant increase in vascular density in CAM. This was confirmed by significant increase in hemoglobin (Hb) content in CAM. Treatment with PPAR γ agonist GW 1929 along with AGE-BSA did not cause any significant change in vascular density in CAM when compared to control. But, PPAR γ antagonist GW 9662 along with AGE reversed the effect of AGE and decreased the microvessel density in CAM; Hb content in CAM decreased significantly (Fig. 1A & B).

Fig. 1—Effect of PPAR γ antagonist and agonist on AGE-BSA induced angiogenesis in chick CAM [8-days-old chick embryo CAMs were treated with non-glycated BSA (control), AGE-BSA, AGE + PPAR agonist GW 1929 (5 µM), AGE + PPAR antagonist GW 9662 (5 µM). The CAMs were isolated on 12th day and observed the capillary network formed around the filter discs. Photograph of new vessels in the CAM (A). Hb content of CAM was determined (B). The results represent average of quadruplicate experiments. *P<0.05 when compared to control. †P<0.05 when compared to AGE]

Fig. 1—Effect of PPAR γ antagonist and agonist on AGE-BSA induced angiogenesis in chick CAM [8-days-old chick embryo CAMs were treated with non-glycated BSA (control), AGE-BSA, AGE + PPAR agonist GW 1929 (5 µM), AGE + PPAR antagonist GW 9662 (5 µM). The CAMs were isolated on 12th day and observed the capillary network formed around the filter discs. Photograph of new vessels in the CAM (A). Hb content of CAM was determined (B). The results represent average of quadruplicate experiments. *P<0.05 when compared to control. †P<0.05 when compared to AGE]
Effect of PPAR γ antagonist and agonist on expression of CD 31 and E-selectin by HUVECs

To further examine the involvement of PPAR γ in AGE-mediated angiogenesis, expression of certain endothelial markers was analyzed in HUVECs in culture. Treatment of HUVECs in culture with AGE-BSA caused significant increase in the levels of both CD 31 and E-Selectin. Treatment with PPAR γ agonist GW 1929 along with AGE-BSA did not cause any significant change in CD 31 and E-selectin levels, while PPAR γ antagonist GW 9662 reversed the effect of AGE-BSA significantly and down-regulated the expression of CD 31 and E-selectin levels (Fig. 2A & B). Significant decrease in E-selectin production by cells treated with AGE + GW 9662 was further confirmed by Western blot (Fig. 3).

Effect of PPAR γ antagonist and agonist on VEGF production

VEGF being an endothelial cell-specific angiogenic growth factor that can exert autocrine as well as paracrine effects, the possibility of AGE-BSA effect involving the modulation of VEGF action was examined. Consistent with the earlier data, treatment of cells with AGE-BSA increased the production of VEGF. ELISA showed reversal of AGE-BSA mediated upregulation of VEGF by PPAR γ antagonist GW 9662. This was further confirmed by Western blot which showed significant decrease in VEGF production in HUVECs treated with AGE + GW 9662. Expression of VEGF was also examined at the gene level by RT-PCR. HUVECs treated with AGE showed significant expression of VEGF and use of PPAR agonist did not cause much variation. But, PPAR antagonist along with AGE significantly decreased VEGF mRNA level also (Figs 4 & 5).

Fig. 2—Effect of PPAR γ antagonist and agonist on expression of CD 31 and E-selectin by HUVECs [HUVECs were maintained in culture in MCDB 131 medium with and without AGE-BSA in presence of PPAR γ agonist GW 1929 (5 µM) and antagonist GW 9662 (5 µM). The levels of cell associated CD 31 (A) and E-selectin (B) secreted into the medium were estimated by ELISA using specific antibodies. Cells maintained in culture supplemented with non-glycated albumin served as control. Values are mean of 5 experiments ± SEM. *P≤0.05 when compared to control. †P≤0.05 when compared to AGE]
Effect of AGE on expression of PPARγ gene in HUVECs

To further examine the involvement of PPARγ in AGE-mediated angiogenesis, expression of PPARγ gene in HUVECs was analyzed and the results are shown in Fig. 6. Treatment with AGE caused upregulation in the expression of PPARγ gene compared to untreated cultures.

Discussion

Data presented here confirmed our earlier results and those reported from other laboratories on the proangiogenic effect of AGE-BSA in different model systems, such as CAM, aortic rings and HUVECs in culture. In chick CAM assay, treatment with AGE-BSA caused significant increase in vascular density in CAM, suggesting proangiogenic effect of AGE in vivo. In HUVECs in culture, treatment with AGE caused faster acquisition of angiogenic phenotype and significantly high expression of angiogenic markers.

Further investigations to study the molecular mechanism of proangiogenic effect of AGE-BSA showed that AGE-mediated angiogenesis involved PPARγ-dependent pathway, as evidenced by the following observations: (i) In chick CAM assay, PPARγ antagonist GW 9662 reversed the AGE-induced increase in vascular density. This was consistent with the earlier report that PPAR agonist induces angiogenesis in a murine corneal model in vivo. However, in our study PPARγ agonist GW 1929 along with AGE-BSA did not cause much variation in CAM density, probably because AGE might have activated the pathway to such an extent that no further activation by agonist took place, suggesting the involvement of PPARγ in AGE-mediated angiogenesis in vivo.

(ii) in HUVECs in culture, effect of AGE-BSA on the expression of angiogenic phenotype was reversed by PPARγ antagonist GW 9662. AGE-BSA increased the expression of angiogenic markers CD 31 and E-selectin. But, treatment with PPARγ antagonist significantly inhibited the expression of these markers, further suggesting PPARγ-dependent downstream pathway in AGE-mediated angiogenesis. However, PPARγ agonist along with AGE did not cause any further effect on the expression of these markers, (iii) the involvement of PPARγ was further evident from increase in mRNA level of PPARγ in HUVECs treated with AGE-BSA. RT-PCR analysis
revealed that AGE treatment caused significant up-regulation of PPARγ mRNA. These results from PPARγ gene expression and reversal of the effect of AGE-BSA by antagonist of PPARγ and the earlier reports on the angiogenic effect of PPARγ ligands thus suggested that the angiogenic effect of AGE involved PPARγ-dependent pathway.

Results of further investigations to understand the mechanism of PPARγ-dependent effect of AGE suggested that it involved modulation of expression of VEGF, an endothelial cell-specific mitogen. AGE-BSA appeared to up-regulate expression of VEGF, which is an endothelial cell-specific growth factor critically involved in exerting both autocrine and paracrine effects, inducing angiogenic phenotype in endothelial cells. PPARγ antagonist reversed the effect of AGE-BSA and significantly decreased the production of VEGF protein. RT-PCR analysis showed that VEGF mRNA levels were also decreased by PPARγ antagonist. PPARγ agonist along with AGE-BSA, however, did not cause any further effect on the expression of VEGF. These results were consistent with the earlier reports, where the role of PPARγ in VEGF production was also indicated by the up-regulation of VEGF expression by agonist of PPARγ in different model systems. PPARγ may be regulating the expression of VEGF gene, as VEGF gene promoter contains PPAR responsive element (PPRE).

It is not clear how AGE can modulate PPARγ activity. AGE-induced oxidant stress might be one of the factors contributing indirectly to PPARγ activation. Previous reports have also shown that stress causes expression of PPARγ gene in cultured mesangial cells through an oxidant stress-dependent mechanism. Our previous study has shown that AGE-BSA produces oxidant stress in HUVECs in culture and antioxidants, such as N-acetyl cysteine and ascorbic acid reverse the effect of AGE on angiogenesis. The oxidant stress generated by AGEs may cause oxidation of several biological molecules, including metabolites of linoleic acid. As several oxidative metabolites of linoleic acid, including 13-hydroxyoctadecadienoic acid (13-HODE) and 13-oxooctadecadienoic acid (13-OXO) have been shown to bind PPAR, they might have acted as ligands for PPARγ and indirectly mediated AGE effect on PPARγ. Another possibility for AGE effect on PPARγ was that AGE products might act as ligands for PPARγ. AGE proteins are endocytosed and partially degraded to small reactive AGE peptides. These peptides may act as ligands for PPAR. Blocking of lysosomal degradation by chloroquine partially reversed the effect of AGE on angiogenic markers in HUVECs, suggesting that lysosomal degradation of AGE was a key process (data not shown). Use of PPARγ antagonist might block any of these mechanisms, causing the reversal of AGE effect.

Many of the diabetic complications are associated with angiogenesis. As AGE-mediated effects on angiogenesis involve PPARγ pathway, the use of drugs that affect PPARγ for treatment of diabetes requires careful consideration. As PPARγ activation induces VEGF expression and angiogenesis, such drugs might be useful in the repair/neovascularization mechanisms at the site of myocardial infraction and in diabetic wounds. But, use of such drugs may produce an opposite effect in the case of diabetic retinopathy.

Acknowledgments
Financial assistance in the form of SRF from UGC, Government of India to MDS is gratefully acknowledged.

References
24


