Abstract—Nonenzymatic glycation is increased in diabetes. The role of advanced glycation end products has been implicated in many of the complications of diabetes, whereas the effects of early-glycation Amadori-modified proteins on vascular cells are poorly defined. In the present study, we show that glycated serum albumin (GSA) induces a parallel activation of the redox-responsive transcription factors (nuclear factor \( \kappa B \)) and AP-1 and increases activity of mitogen-activated protein kinases (MAPKs), extracellular signal-regulated kinase (ERK), and p38 MAPK in vascular smooth muscle cells (VSMCs). GSA increased expression of early response genes, \( c-fos \) and \( c-jun \), and inflammatory genes, monocyte chemoattractant peptide (MCP-1), and interleukin (IL)-6. These effects were comparable to bacterial lipopolysaccharide, tumor necrosis factor-\( \alpha \), (TNF-\( \alpha \)), IL-1\( \beta \), angiotensin II, epidermal growth factor, and the phorbol ester PMA. One of signaling pathways by which GSA activates VSMCs appears to be via nuclear factor \( \kappa B \) activation, leading to induction of MCP-1 and IL-6 gene expression, comparable to the effects of lipopolysaccharide, TNF-\( \alpha \), and IL-1\( \beta \). Another signaling cascade by which GSA activates VSMCs is the ERK\( \rightarrow c-Fos \rightarrow AP-1 \) pathway, which may lead to stimulation of cell proliferation and migration. These effects are comparable to the effects of angiotensin II, epidermal growth factor, and PMA. Incubation of VSMCs with the antioxidant N-acetylcysteine suppressed GSA-elicited mRNA induction of MCP-1 and IL-6. Inhibition of p38 MAPK but not ERK caused attenuation of MCP-1 and IL-6 mRNA induction. Finally, GSA caused a significant stimulation of VSMC growth and migration. These findings suggest that GSA may play a role in diabetic atherogenesis by activating VSMCs, leading to induction of inflammatory mediators in the vessel wall, as well as proliferation and migration of VSMCs. (Hypertension. 2002;39:22-28.)

Key Words: glycation | albumin | inflammation | proliferation | migration | muscle, smooth, vascular

Accelerated atherosclerosis and microvascular disease are the major vascular complications of diabetes and constitute the principal cause of morbidity and mortality in this common disorder.\(^1,2\) Whereas many underlying factors potentially contribute to the development of diabetic vasculopathy, including abnormalities in plasma lipoproteins, blood pressure, and renal function, hyperglycemia is generally believed to be a major causative factor. Among various theories that have been proposed to explain the hyperglycemia-induced pathogenesis of vascular complications of diabetes, a growing body of evidence suggests that many effects are mediated by nonenzymatic glycation products.\(^3,4\) Nonenzymatic glycation is a condensation reaction between glucose and reactive protein amino acid groups, yielding Schiff base intermediates that undergo Amadori rearrangement to form stable protein-glucose adducts.\(^5,7\) Further irreversible chemical reactions of these intermediate glycated Amadori products may lead to the formation of advanced glycation end products (AGEs).

Serum levels of AGEs are increased in diabetics and have been implicated in many of the complications of diabetes. To date, most studies have focused on the relationship between vascular complications and AGEs, while only a few studies have been performed to assess the role of early glycation products. In animal studies, elevated concentrations of Amadori albumin promote a generalized vasculopathy and have been implicated in the development of diabetic nephropathy and retinopathy.\(^8-11\) A recent clinical study has clearly shown an association between Amadori albumin and diabetic vascular complications.\(^12-14\)

AGEs exert their effects on cells by interacting with specific cellular receptors, the best characterized of which is the receptor for AGE (RAGE).\(^15,16\) It has been demonstrated that AGE-RAGE interaction-mediated induction of cellular oxidant stress triggers a cascade of intracellular signals involving p21\(^*\)* and mitogen-activated protein kinase (MAPK), culminating in transcription factor activation.\(^17\)

Early glycation products such as Amadori albumin may also exert their effects through specific cellular receptors and activation of signaling pathways. Therefore, we sought to define the signaling pathways activated by glycated serum albumin (GSA; Amadori adducts of serum albumin) in vascular smooth muscle cells (VSMCs).

In the present study, we investigated whether GSA delivers signals to induce the activation of the redox-responsive

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transcription factors nuclear factor κB (NF-κB) and AP-1, because GSA may contribute to atherosclerosis through redox-sensitive signaling events. We also examined the ability of GSA to activate extracellular signal-regulated kinase (ERK), a subgroup of the MAPK family, which has been shown to be a critical regulatory factor for growth factor–mediated migration and growth and may act as an intermediate in the cascade of events that regulate NF-κB and AP-1 activation. Therefore, we investigated whether GSA induces mRNA expression for monocyte chemoattractant peptide (MCP-1) and interleukin (IL)-6, as well as the functional relationship between induced gene expression and activated signaling pathways. The effects of GSA on VSMC growth and migration were also evaluated.

Methods

Cell Culture and RNA Extraction

Rat VSMCs were isolated by elastase and collagenase digestion of thoracic aortae from male Wistar rats. Total RNA was extracted from confluent VSMCs by use of a modified guanidinium isothiocyanate method.

NF-κB and AP-1 Activation

The cells were stably transfected with a cis-reporter plasmid containing the luciferase reporter gene linked to 5 repeats of NF-κB binding sites (pNFκB-Luc, Stratagene) or to 7 AP-1 sites (pAP-1-Luc, Stratagene). Several clones were selected for analysis of NF-κB and AP-1 activation.

MAPK Assay

We used a transreporting system with a fusion transactivator plasmid (Stratagene) that consists of the DNA binding domain of the yeast GAL4 (residues 1 to 147) protein and the activation domain of Elk-1, c-Jun, or Chop, respectively. The clones that gave the best response when transfected with the positive control vectors for each reporting system were then subjected to experimental analysis.

Analysis of mRNA Expression

Probes were obtained by reverse transcription-polymerase chain reaction (RT-PCR) with specific primers for MCP-1 and IL-6, labeled with [α-32P]dCTP by random priming, and used for Northern blot analysis of mRNA expression. mRNA expression of MCP-1, IL-6, c-fos, and c-jun was also analyzed by RT-PCR with specific primers for c-fos and c-jun designed according to published sequences.

Cell Proliferation

To assess cell proliferation, Alamar Blue (10 wt%/vol% in PBS, Serotec Ltd) was added to the cells, and 3 hours later, fluorescence was determined by use of excitation/emission wavelengths of 544/590 nm in a cytofluorometer (Fluoroskan Asent FL).

VSMC Migration

VSMCs were added to 24-well inserts (pore size, 8 μm) that demonstrate fluorescence blocking capability (FALCON HTS FluoroBlok, Becton Dickinson) at a density of 50,000 cells per insert. DMEM with 1 mg/mL GSA was used in the lower wells, whereas DMEM/5% nonglycated serum albumin (1 mg/mL) was added to the control (lower) wells. Then, fluorescence of cells that had migrated through the inserts was measured on the cytofluorometer in the bottom read mode with excitation/emission wavelengths of 485/530 nm.

Materials

Human serum albumin (nonglycated and glycated) was obtained from Sigma Chemical Co. GSA contains 3 moles of fructosamine per mole of albumin. The GSA preparation did not contain contaminant bacterial endotoxin (lipopolysaccharide [LPS]) or measurable AGEs as determined by fluorescence assays (from 360 to 600 nm) on excitation at 370 nm or 350 nm. The ~35-kDa extracellular domain of the rat cellular receptor for AGEs, called soluble RAGE (sRAGE), was kindly provided by Dr Y. Kawakami (Tsukuba University School of Medicine). LPS and phorbol ester myristate (PMA) were purchased from Sigma. Tumor necrosis factor-α (TNF-α), IL-1β, and epidermal growth factor (EGF) were purchased from Peprotech, Inc., and angiotensin II (Ang II) was from Pepptide, Inc.

Statistical Analysis

Data are presented as mean±SEM. Multiple comparisons were evaluated by ANOVA, followed by Fisher’s protected least-significant-difference test. Student’s unpaired t test was used for comparisons between 2 experiments. A value of P<0.05 was considered statistically significant.

An expanded Methods section can be found in an online data supplement available at http://www.hypertensionaha.org

Results

NF-κB and AP-1 Activation

We examined the GSA inducibility of NF-κB–mediated reporter gene expression in VSMCs comparing with LPS, TNF-α, IL-1β, Ang II, EGF, and PMA. LPS, TNF-α, and IL-1β potently stimulated NF-κB–mediated gene transcription in VSMCs. Although Ang II and PMA weakly induced NF-κB–mediated gene transcription, EGF had very little effect. GSA substantially activated NF-κB–mediated gene transcription with potency comparable to IL-1β. NF-κB–dependent transactivation increased 13.6-fold relative to unstimulated levels in GSA-treated VSMCs at a concentration of 1 mg/mL (Figure 1A). The inset in Figure 1A shows the dose dependence of NF-κB activation by GSA. In contrast, nonglycated serum albumin was totally ineffective (data not shown). Similarly, we examined whether GSA induces AP-1–mediated reporter gene expression in VSMCs compared with LPS, TNF-α, IL-1β, Ang II, EGF, and PMA. LPS, TNF-α, and IL-1β had little effect, whereas Ang II and PMA potently and EGF less potently but significantly stimulated AP-1–mediated gene transcription in VSMCs (Figure 1B). GSA activated AP-1–mediated gene transcription with a potency comparable to EGF. AP-1–dependent transactivation increased 1.75-fold relative to unstimulated levels in GSA-treated VSMCs at a concentration of 1 mg/mL (Figure 1B). The inset in Figure 1B shows the dose dependence of AP-1 activation by GSA. In contrast, nonglycated serum albumin had little effect (data not shown). As shown in Figure 1C, AGE (100 μg/mL) induced activation of NF-κB or AP-1 with a potency comparable to GSA (1 mg/mL). Cotreatment of the cells with increasing concentration of an sRAGE significantly suppressed AGE-induced NF-κB or AP-1 activation but did not affect NF-κB or AP-1 activation by GSA.

MAPK Activation

We next examined the effect of GSA on the activation of MAPK pathways. For this, activation of the transcription factors Elk-1, c-Jun, or Chop was evaluated with a transreporting system by use of GAL4 fusion transactivators as...
Figure 1. A, Effects of GSA and control agents on NF-κB–dependent transcriptional activity. Quiescent VSMCs (transfected with pNFκB-Luc) were left untreated (Cont) or were treated with LPS 30 μg/mL, TNF-α 10 ng/mL, IL-1β 10 ng/mL, Ang II 100 nmol/L, EGF 100 ng/mL, PMA 100 nmol/L, or GSA 1 mg/mL. After 4 hours, cells were lysed, and luciferase activities were measured. Inset, Cells were treated with different concentrations of GSA (0.003 to 1 mg/mL) for 4 hours, after which luciferase activities were measured. B, Effects of GSA and control agents on AP-1–dependent transcriptional activity. Quiescent VSMCs (transfected with pAP-1-Luc) were left untreated (Cont) or were treated with LPS 30 μg/mL, TNF-α 10 ng/mL, IL-1β 10 ng/mL, Ang II 100 nmol/L, EGF 100 ng/mL, PMA 100 nmol/L, or GSA 1 mg/mL. After 4 hours, cells were lysed, and luciferase activities were measured. Inset, Cells were treated with different concentrations of GHS (0.003 to 1 mg/mL) for 4 hours, after which luciferase activities were measured. C, Effects of sRAGE on GSA– or AGE-mediated NF-κB or AP-1 activation. Quiescent VSMCs (transfected with pNFκB-Luc or pAP-1-Luc) were pretreated with an excess of sRAGE (20-molar excess to GSA or AGE) for 30 minutes and then stimulated with GSA 1 mg/mL or AGE 100 μg/mL. After 4 hours, cells were lysed, and luciferase activities were measured. Data are mean±SEM of triplicate observations. *P<0.05, **P<0.01 vs control (medium only).
pathway-specific sensors. When activated by phosphorylation, these fusion proteins bind to the promoter and induce luciferase expression. Therefore, luciferase activity in stable cell lines reflects the activation status of the fusion transactivator and hence the activation status of corresponding signal pathways. GSA substantially increased Elk-1 activity and, to a lesser extent, Chop activity (Figure 2A). GSA also modestly but significantly increased c-Jun activity (Figure 2A). The increase in Elk-1 activity by GSA appears to reflect the activation of ERK in VSMCs. In fact, GSA-stimulated Elk-1 activity in this system was completely prevented by the MAPK kinase (MEK) inhibitor PD98059 (inset in Figure 2A).

We examined the ability of GSA to activate Elk-1 compared with LPS, TNF-α, IL-1β, Ang II, EGF, and PMA. LPS, TNF-α, and IL-1β weakly, Ang II and EGF moderately, and PMA markedly stimulated Elk-1 activity (Figure 2B). GSA potently induced Elk-1 activation at a level comparable to PMA (Figure 2B). In contrast, nonglycated serum albumin had no significant effect on Elk-1 activation (data not shown).

mRNA Expression

We next examined the effect of GSA on the mRNA levels of MCP-1, IL-6, c-Fos, and c-Jun in VSMCs by RT-PCR. LPS, TNF-α, and IL-1β potently increased gene expression for MCP-1 and IL-6, whereas Ang II, EGF, and PMA to a lesser extent upregulated mRNA levels for MCP-1 and IL-6. GSA substantially increased mRNA levels for MCP-1 and IL-6, comparable to LPS, TNF-α, and IL-1β (Figure 3A). In contrast, c-fos gene expression was induced by Ang II, EGF, and PMA, but LPS, TNF-α, and IL-1β had little effect. GSA induced c-fos gene expression comparable to Ang II, EGF, and PMA. GSA also upregulated c-jun gene expression in VSMCs. Control PCR experiments demonstrated equivalent amounts of GAPDH mRNA in all samples. Nonglycated serum albumin had no significant effect on the expression of all 4 genes examined (data not shown).

We further characterized the ability of GSA to induce gene expression of MCP-1 and IL-6 using signaling pathway inhibitors. Northern blot analysis showed that a basal level of MCP-1 mRNA is present in untreated VSMCs. After treatment with GSA, MCP-1 mRNA substantially increased within 2 hours and remained elevated for 24 hours (Figure 3B). IL-6 mRNA was expressed at very low levels in untreated VSMCs but increased within 2 hours after treatment with GSA, with a subsequent decrease by 8 hours and an increase by 24 hours (Figure 3C). The effects of the MAPK inhibitors PD98059 and SB203580 or an antioxidant, N-acetylcysteine (NAC) on GSA-induced increases in mRNA levels of MCP-1 and IL-6 were evaluated in VSMCs treated with GSA for 4 hours. As shown in Figure 3B, GSA-elicited MCP-1 mRNA expression was not affected by PD98059, but it was markedly suppressed by SB203580. GSA-induced MCP-1 mRNA levels were inhibited by NAC. GSA-elicited IL-6 mRNA expression was also not affected by PD98059, but it was prevented by SB203580. GSA-induced IL-6 mRNA levels were also potently inhibited by NAC (Figure 3C).

Cell Proliferation and Migration

Finally, we examined whether GSA stimulates VSMC growth and migration. After exposure of growth-arrested VSMCs to GSA, cell proliferation was determined by the Alamar-blue assay after 2 days of treatment. Fluorescent values, as an indicator of viable cell number, were significantly increased after treatment with GSA in a dose-dependent manner (Figure 4A). In contrast, nonglycated...
albumin had little effect. When cell migration was assessed by analyzing cell motility through membrane pores, GSA significantly increased migration activity compared with nonglycated albumin (Figure 4B).

Discussion
Patients with diabetes mellitus have a marked propensity for cardiovascular morbidity and mortality. VSMCs may play an influential role in the progression of diabetic macroangiopathy. In atherosclerosis, VSMCs may be activated in response to various stimuli to migrate from the media to the intima of the aorta and proliferate. The biological effects of Amadori adducts on VSMCs may be involved in such diabetic atherogenesis.

In the present study, we demonstrated that GSA is a potent stimulator of NF-κB activation. This potency was comparable to that of bacterial LPS or inflammatory cytokines such as TNF-α or IL-1β, whereas growth factors such as Ang II or EGF and the phorbol ester PMA were much less potent in inducing NF-κB activation. The transcription factor NF-κB has an important function in the regulation of a variety of genes involved in the inflammatory and proliferative responses of cells, and recent studies strongly indicate that NF-κB is involved in the pathogenesis of atherosclerosis.30–32 We found that GSA induces gene expression for MCP-1 and IL-6 in VSMCs. Induction of these mRNAs was also observed in response to LPS, TNF-α, or IL-1β, whereas Ang II, EGF, and PMA were weak inducers of these genes. This observation suggests that the induction of MCP-1 and IL-6 gene expression by GSA is mediated primarily by NF-κB activation. Thus, one of the signaling pathways by which
GSA activates VSMCs appears to be via NF-κB activation. We have also demonstrated that GSA is a modest stimulator of AP-1 activation. LPS, TNF-α, and IL-1β were unable to induce AP-1 activation, whereas Ang II, EGF, and PMA substantially induced AP-1 activation. We also found that GSA is a potent stimulator of ERK. PMA is a very potent activator of ERK. EGF and Ang II substantially induced ERK activation, whereas LPS, TNF-α, and IL-1β were less potent. The present observation suggests that the inducibility of these agents for ERK activation may be functionally related to c-fos gene expression and AP-1 activation in GSA-stimulated VSMCs. ERK could lead to the induction of c-fos gene expression. Being the major component of the AP-1 transcription factor, c-Fos, once activated together with c-Jun, can subsequently activate the transcription of several genes controlling cellular growth. This could be the case in GSA-stimulated VSMCs. Thus, another signaling pathway for GSA to activate VSMCs appears to be the ERK→c-fos→AP-1 pathway.

MCP-1, which is chemotactic for monocytes both in vitro and in vivo, has been detected in atherosclerotic lesions from both human and experimental animals but not in normal arteries, suggesting that it may play a significant role in the pathogenesis of atherosclerosis. IL-6, which is a multifunctional cytokine that mediates B-lymphocyte proliferation/induction of antibody synthesis and mediates the hepatic acute-phase response, also plays an important role in the vessel wall as a VSMC growth factor through a paracrine mechanism. Because GSA may contribute to atherogenesis in part through induction of MCP-1 and/or IL-6, we further examined the ability of GSA to induce the expression of these genes and the signal events involved. Thiol antioxidants have been shown to inhibit MCP-1 gene expression in response to a variety of proinflammatory stimuli in endothelial cells. In the present study, NAC inhibited the induction of MCP-1 gene expression by GSA in VSMCs, suggesting that the regulation of MCP-1 gene expression by GSA occurs through redox-sensitive transcriptional mechanisms. The finding that MCP-1 gene expression is induced in VSMCs in response to such agents that potently induce NF-κB activation suggests that MCP-1 gene expression induced by GSA is at least partly NF-κB dependent. IL-6 gene expression by Ang II has been shown to be regulated through NF-κB activation by GSA is at least partly NF-κB dependent. IL-6 gene expression by Ang II has been shown to be regulated through NF-κB activation. In this study, we observed that those stimuli that activate NF-κB more potently than Ang II increased the levels of IL-6 to a greater extent than Ang II did. Consistent with this observation, we have also observed that NAC inhibited IL-6 gene expression in VSMCs, suggesting that the induction of IL-6 expression by GSA may be also at least partly NF-κB dependent. MAPKs are a family of serine/threonine protein kinases activated as early responses to a variety of stimuli involved in cellular growth, transformation, and differentiation. They are also involved in activation of AP-1 and NF-κB. GSA markedly activated ERK and also significantly activated p38 MAPK in VSMCs. Using the MAPK kinase (MEK) inhibitor PD 098059 and the p38 MAPK inhibitor SB203580, we demonstrated that GSA-induced MCP-1 and IL-6 mRNA expression occurs through the p38 MAPK-sensitive but ERK-insensitive pathway. On the other hand, marked activation of ERK by GSA appeared to lead to AP-1 activation and stimulation of cell proliferation. Indeed, PD98059 prevented GSA-stimulated VSMC proliferation by inhibiting GSA-elicited activation of ERK and AP-1 in our previous study.

This study suggests the importance of glycated Amadori products in atherogenesis in diabetics by showing that GSA stimulates VSMC growth and migration. To evaluate whether the authentic Amadori adducts exert similar biological effects on VSMCs, we purified glycated albumin from human albumin (human albumin, Fraction V Powder, Sigma) using affinity chromatography in aminophenyl boronate gels and examined its ability to activate VSMCs. We found that human albumin contains unexpectedly high levels of glycated albumin and that this authentic glycated albumin also activates NF-κB and AP-1 in VSMCs (unpublished observation). In the present study, the concentrations of GSA that produced half-maximal stimulation of NF-κB and AP-1 in VSMCs were ≈200 μg/mL. These values were well below the normal plasma concentration range of GSA (350 to 500 μg/mL), and at concentrations exceeding the normal plasma range, GSA potently activated VSMCs. Because of the close correlation between serum glucose content and the degree of albumin glycation, the concentrations at which GSA could induce VSMC activation are likely to be easily reached in diabetics. The GSA used in our study was purified to exclude residual contamination with AGEs. Moreover, treatment of the cells with a soluble form of the receptor for AGEs affected neither NF-κB nor AP-1 activation by GSA. These results indicate that the response obtained with GSA is not due to contaminating AGEs. Estimated plasma levels of AGEs were ≈15 μg/mL in diabetics according to a recent report. AGE appears to be a stronger inducer of NF-κB/AP-1 than GSA. Because plasma concentration of AGEs seems much lower than that of GSA, one might estimate overall effects (potency x concentration) in vivo (in plasma) to be almost equal with AGEs and GSA.

Atherosclerosis is now recognized as an inflammatory process. Our study shows that expression of mRNAs for MCP-1 and IL-6 is induced in response to GSA, providing a molecular basis for this induction in VSMCs. We also show that GSA indeed stimulates VSMC growth and migration. These findings suggest that GSA may play a role in diabetic atherogenesis by inducing inflammatory mediators in the vessel wall, as well as its own proliferative and migratory effects on VSMCs. Several important questions await answers. First, GSA activates signals such as inflammatory cytokines, leading to NF-κB activation and induction of inflammatory mediators, and growth factors, leading to ERK activation and cell growth. How can GSA elicit such a broad range of signals? Second, does GSA in diabetic patients indeed exert the same biological effects on VSMCs? Elucidation of GSA receptor(s) and the resultant postreceptor signaling will provide new insights into the molecular mechanisms underlying GSA-mediated proinflammatory and proatherogenic responses that may link GSA to acceleration of diabetic complications. We are currently investigating the chemical characterization of GSA from diabetic patients and its ability to activate VSMCs.
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References


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