Activation of the Receptor for Advanced Glycation End Products Triggers a p21ras-dependent Mitogen-activated Protein Kinase Pathway Regulated by Oxidant Stress*

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Advanced glycation end products (AGEs) exert their cellular effects on cells by interacting with specific cellular receptors, the best characterized of which is the receptor for AGE (RAGE). The transductional processes by which RAGE ligation transmits signals to the nuclei of cells is unknown and was investigated. AGE-albumin, a prototypic ligand, activated p21ras in rat pulmonary artery smooth muscle cells that express RAGE, whereas nonglycated albumin was without effect. MAP kinase activity was enhanced at concentrations of AGE-albumin, which activated p21ras and NF-kB. Depletion of intracellular glutathione rendered cells more sensitive to AGE-mediated activation of this signaling pathway. In contrast, signaling was blocked by preventing p21ras from associating with the plasma membrane or mutating Cys118 on p21ras to Ser. Signaling was receptor-dependent, because it was prevented by blocking access to RAGE with either anti-RAGE IgG or by excess soluble RAGE. These data suggest that RAGE-mediated induction of cellular oxidant stress triggers a cascade of intracellular signals involving p21ras and MAP kinase, culminating in transcription factor activation. The molecular mechanism that triggers this pathway likely involves oxidant modification and activation of p21ras.

In the presence of aldoses, proteins become nonenzymatically glycated and oxidized (1–3). This initially reversible glycation is followed by further irreversible rearrangements leading to a class of permanently modified proteins known as advanced glycation end products (AGEs).1 Although glycated proteins are found at low levels in normal individuals during aging, significantly higher levels are found in certain disease states such as diabetes and renal failure (4, 5). We have identified a cellular receptor for AGEs, termed RAGE, which exhibits a wide tissue distribution (6–9). We have recently demonstrated the enhanced presence of RAGE in vascular smooth muscle of diabetic vasculature (renal arterial vessel) compared with a similar sized vessel from a nondiabetic age-matched control. These areas of enhanced RAGE immunoreactivity colocalize with enhanced immunostaining for AGE-reactive epitopes (10). Our previous data in endothelial cells and in vivo demonstrated that interaction of AGEs with RAGE results in triggering a range of cellular responses, including transcription factor activation and changes in gene expression (11–14). However, the means by which a signal reflecting AGE engagement of RAGE is transmitted to the nucleus is not known. Given the enhanced expression of AGE and RAGE in diabetic vascular smooth muscle, we focused on elucidating the signaling pathways in smooth muscle cells that are triggered upon ligation of RAGE by AGE-albumin, a prototypical ligand.

Recent evidence supports a role for reactive oxygen species in mediating signaling by several receptor systems (15–21). For example, platelet-derived growth factor has recently been shown to stimulate H2O2 production in vascular smooth muscle cells (15). When production of oxidants was blocked, platelet-derived growth factor-induced enhancement of mitogen-activated protein (MAP) kinase activity, chemotaxis, and DNA synthesis was prevented (15). Others have found that induction of c-fos expression by tumor necrosis factor-α and basic fibroblast growth factor requires production of reactive oxygen intermediates (16), as does activation of the MAP kinase cascade in NIH-3T3 cells (17) and in neutrophils (18).

Our previous data indicate that interaction of AGEs with endothelial RAGE and vascular RAGE in vivo results in the generation of significant cellular redox stress manifested by the appearance of malondialdehyde-reactive epitopes, increased mRNA for heme oxygenase-1 (12) and activation of the transcription factor NF-κB (12). Furthermore, we have recently identified a p21ras/MAP kinase signaling cascade that is regulated by redox stress (22). Therefore, we performed studies to elucidate the molecular signals that result from AGE-RAGE interaction.

EXPERIMENTAL PROCEDURES

Materials—The farnesyltransferase inhibitor α-hydroxyfarnesylphosphonic acid was obtained from Boiroml (Plymouth Meeting, PA), and l-buthionine-(S,R)-sulfoximine was from Sigma.

Preparation of Proteins—Bovine serum albumin (Sigma) was glycated by incubation with glucose (0.5 mM) or ribose (0.25 mM) at 37 °C for 6 weeks (6, 7). Controls consisted of the same initial preparations of albumin incubated in the same manner in the absence of aldose. Rat RAGE and nonspecific rabbit-anti-RAGE IgG were prepared and characterized as described previously for bovine RAGE (6, 12). The ~35 kDa extracellular domain of rat RAGE was termed soluble RAGE (sRAGE).

Cell Culture and Treatment—Rat pulmonary artery smooth muscle cells and PC12 cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum (and 5% horse serum for PC12 cells) and 2% l-glutamine. Cells treated with inhibitors were

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1 The abbreviations used are: AGE, advanced glycation end products; RAGE, receptor for AGE; sRAGE, soluble RAGE; SMC, rat pulmonary artery smooth muscle cell; MAP, mitogen-activated protein.
RESULTS

Endogenous p21\(^{ras}\) Is Activated by AGEs—Our previous studies have revealed activation of p21\(^{ras}\) by reactive oxygen and reactive nitrogen species (22, 25). Because the ability of AGEs to induce NF-\(\kappa\)B activation has been shown to be dependent upon the generation of cellular redox stress (12), we examined whether AGEs initiate signaling to the nucleus by activating p21\(^{ras}\). Addition of AGE-albumin (100 \(\mu\)g/ml) to rat pulmonary artery smooth muscle cells (SMCs), in which the intracellular nucleotide phosphate pools were labeled to equilibrium with \(\[^{32}\text{P}\] \text{orthophosphate, yielded enhanced levels of GTP-bound p21}^{\text{ras}}\) upon immunoprecipitation (Fig. 1, solid circles). In contrast, cells treated with nonglycated albumin (Fig. 1, open circles and open triangles) had p21\(^{ras}\) with basal levels of GTP associated with it (13.2 ± 2.5%). Furthermore, cells in which endogenous glutathione levels were greatly reduced by pretreatment with \(\text{l-buthionine-(S,R)-sulfoximine (BSO)}\) (20, 22) showed an enhanced response on subsequent addition of AGE-albumin (Fig. 1, solid triangles). We have previously demonstrated that this concentration of AGE-albumin was optimal for AGE-RAGE-mediated cellular oxidant stress (12). These data indicate that endogenous p21\(^{ras}\) was stimulated by addition of AGE and that this activation is under redox regulation.

MAP Kinase Activation by AGEs—One of the known critical intermediates in signal transduction by p21\(^{ras}\) is activation of a family of Ser/Thr kinases termed MAP kinases. We investigated whether two of these kinases, ERK1 and ERK2, were activated by AGE-albumin. Treatment of SMCs with AGE-albumin for 10–15 min resulted in concentration-dependent activation of immunoprecipitated ERK1 and ERK2 (Fig. 2, open circles). Addition of nonglycated albumin had no effect (data not shown). Depletion of glutathione by exposure of SMCs to \(\text{l-buthionine-(S,R)-sulfoximine enhanced AGE-albumin stimulation of MAP kinase activity (Fig. 2, solid circles). We have previously reported an increase of 1.5–2-fold in MAP kinase activity upon addition of free radical generators (22).}

Fig. 1. Effect of AGE-albumin concentration on p21\(^{ras}\) activity. Cells with nucleotide pools preloaded with \(\[^{32}\text{P}\]\) were treated with either normal albumin (N-Albumin) or AGE-modified albumin (AGE-Albumin). Some cells were also pretreated for 24 h with \(\text{l-buthionine-(S,R)-sulfoximine (BSO)}\). After 10 min, cells were lysed, and the GTP to GDP ratio of p21\(^{ras}\) was analyzed (see "Experimental Procedures"). Basal levels of GTP-p21\(^{ras}\) were 13.2 ± 2.5. Data points represent the means and standard deviation of at least three independent experiments, each performed in duplicate.

Fig. 2. Effect of AGE-albumin concentration on MAP kinase activity. Cells were treated with the indicated concentrations of AGE-albumin for 10–15 min prior to lysis, and analysis of ERK1 and ERK2 activities was performed as described. Some cells were also pretreated for 24 h with \(\text{l-buthionine-(S,R)-sulfoximine (BSO)}\). Data points represent the means and standard deviation of at least three independent experiments, each performed in duplicate.

Fig. 3. Kinetics of MAP kinase activation by AGE-albumin. Cells were treated for the indicated times with AGE-albumin (100 \(\mu\)g/ml) prior to lysis and analysis of ERK1 and ERK2 activities was performed as described. Data points represent the means and standard deviation of at least three independent experiments, each performed in duplicate.
 Although the amplitude of stimulation seen in our experiments was very consistent, others have found greater MAP kinase activation with different stimuli and cells. This is likely due to the relatively high level of basal MAP kinase activity in our SMC cultures compared with other systems in which basal activity is minimal.

Activation of MAP kinases is typically an early event in cellular activation. Therefore, we investigated the time course of AGE-RAGE-mediated activation of MAP kinase. Peak activity was observed by 10 min after exposure of cells to AGE-albumin (100 μg/ml) (Fig. 3). MAP kinase activity increased as early as 2 min and returned to basal levels at 60 min. Thus, stimulation of ERK1 and ERK2 activities by AGE-albumin resulted in an early, transient stimulation of MAP kinase activity.

Receptor Dependence of AGE Stimulation—Generation of free radicals and cellular oxidant stress has been attributed to AGEs themselves (26, 27) and as a consequence of their interaction with RAGE (12, 13). Therefore, we investigated whether AGE interaction with RAGE on SMC was necessary for MAP kinase activation. Receptor blockade was achieved by the addition of sRAGE, a truncated form consisting of only the extracellular domain of the receptor, or non-AGE-specific, polyclonal anti-RAGE IgG (Fig. 4). Addition of increasing concentrations of sRAGE prevented AGE-enhanced MAP kinase activity in a concentration-dependent manner (Fig. 4, open bars). One likely explanation for the effect of sRAGE lies in its ability to bind exogenous AGEs, thereby inhibiting their interaction with cell surface RAGE and preventing generation of oxidant stress. Anti-RAGE IgG, previously shown to prevent AGE binding to RAGE (6), also prevented AGEs from stimulating MAP kinase activity in SMCs in a concentration-dependent manner (Fig. 4, hatched bars). In contrast, non-immune IgG was without effect (data not shown). Thus, activation of the MAP kinase cascade required AGE-RAGE interaction at the cell surface.

The Role of p21<sup>ras</sup> in the Signaling Cascade—The critical test of our hypothesis involved determining whether AGE-induced stimulation of p21<sup>ras</sup> was linked to MAP kinase and NF-κB activation in SMC. To evaluate this, cells were pretreated with α-hydroxymethylfarnesylphosphonic acid (an inhibitor of farnesyltransferase). This compound specifically inhibits farnesyltransferase, the enzyme responsible for the lipid modification of p21<sup>ras</sup> (28). Without this lipid, p21<sup>ras</sup> does not associate with the plasma membrane and thus cannot activate its effectors and signal downstream (29). We found that SMC pretreated with this inhibitor no longer responded to AGE-albumin by activating ERK1 and ERK2 kinases (Fig. 5). Furthermore, AGE-mediated nuclear translocation of NF-κB was also prevented by inhibition of farnesyltransferase (Fig. 6).

These data indicate that p21<sup>ras</sup> or a related low molecular weight G protein is required for signal transduction following AGE binding to cell surface RAGE.

A molecular target of reactive free radicals on p21<sup>ras</sup> has been defined as Cys<sup>118</sup> (30). To determine if this site is targeted by reactive oxygen species generated by AGE-albumin, we created a mutant of p21<sup>ras</sup> in which Cys<sup>118</sup> was changed to a Ser (30) and overexpressed it in PC12 cells, which also express RAGE (9). These cells, termed PC12 p21<sup>ras</sup>C118S, were compared with wild-type parental cells for their ability to respond to AGE-albumin by activation of p21<sup>ras</sup>-dependent MAP kinase activity. As seen in Fig. 7, wild-type cells responded to AGE-albumin by activating ERK1/2 kinases, whereas PC12 p21<sup>ras</sup>C118S cells were refractory to AGE-albumin. These data provide a molecular basis for RAGE signal transduction.

**DISCUSSION**

Recently, a role for reactive oxygen intermediates in mediating signal transduction has become apparent. For example, the ability of platelet-derived growth factor to stimulate MAP kinase activity, DNA synthesis, and chemotaxis in vascular smooth muscle cells was completely blocked by the addition of catalase or antioxidants (15). These cells were also found to produce H<sub>2</sub>O<sub>2</sub> upon growth factor addition (15). Others have found that antioxidants blocked the ability of a variety of unrelated stimuli to trigger NF-κB activation (31). Thus, a role for free radicals in mediating signal transduction is emerging.
activity, and NF-κB complex. The arrow denotes migration of the NF-κB-DNA protein complex.

FIG. 6. Effect of farnesyltransferase inhibition on AGE-albumin-induced NF-κB activation. Cells were left untreated (− FT Inh.) or treated with α-hydroxymethylfarnesyl-phosphonic acid (+ FT Inh., 10 μM) for 24 h prior to addition of the indicated concentrations of AGE-albumin. After 4 h, nuclei were isolated and assayed for NF-κB binding activity. The arrow denotes migration of the NF-κB-DNA protein complex.

FIG. 7. MAP kinase activity in wild-type and mutant PC12 cells. Wild-type cells (wt) or cells overexpressing p21ras with a Cys118 to Ser mutation were treated with the indicated concentrations of AGE-albumin for 10 min prior to assay. The means and standard deviation of three experiments are shown.

We have previously demonstrated that interaction of AGES with RAGE induces cellular oxidant stress, probably as a consequence of the generation of reactive oxygen intermediates (11, 12). In this study our aim was to determine if cellular oxidant stress consequent to AGE-RAGE interaction would trigger the p21ras/MAP kinase pathway. Our observations indicate that this is indeed the case because p21ras MAP kinase activity, and NF-κB nuclear translocation are stimulated by AGE-albumin, enhanced by glutathione depletion, and prevented by blockade of RAGE. The molecular mechanism may be due to triggering of p21ras exchange activity by reactive oxygen modification of Cys118 on p21ras. There are likely to be many signaling pathways initiated by AGE-RAGE interaction, but the one outlined herein is apparently important because inhibition of p21ras blocked NF-κB activation. This transcription factor is critical for stimulation of many acute phase response genes, and thus the induction of many genes is likely to be controlled by this pathway. In fact, the heme oxygenase gene is under control of NF-κB (32) and is induced upon AGE-RAGE interaction (12). In addition, our previous work has indicated that AGE-mediated activation of NF-κB results in binding of this transcription factor to specific sites within the promoter of the VCAM-1 gene (33). Enhanced expression of this endothelial cell adhesion molecule alters cellular phenotype, potentially resulting in the adherence of mononuclear phagocytes to the vessel wall (34).

In this work, we have examined signaling pathways likely to be of importance when AGES ligate vascular smooth muscle cell RAGE. One of the consequences of this interaction might be enhanced production of monocyte and smooth muscle cell chemotactant factors (14). Identification of the molecular basis underlying these events, possibly linked to the accelerated vascular disease observed in diabetes, is likely a central step toward understanding the consequences of AGE-RAGE interaction not only in vascular smooth muscle cells but also in diverse cell types such as endothelial cells and mononuclear phagocytes. The present report advances our knowledge of the processes set in motion when AGES bind cellular RAGE, an event that culminates in transcription factor activation and alteration of cellular properties.

Past studies have not identified the source of the reactive oxygen intermediates generated by the AGE-RAGE complex. On one hand, AGES by themselves produce reactive oxygen species that could interact directly or indirectly with critical cellular targets. Alternatively, AGE engagement of RAGE could initiate a series of events resulting in intracellular generation of reactive oxygen species, possibly by a system analogous to that of the NADPH oxidase system of neutrophils. Detailed studies examining these possibilities are ongoing.

AGES are prevalent in certain pathophysiological conditions such as diabetes (4, 5) and renal failure or Alzheimer’s disease, in which delayed protein turnover favors irreversible nonenzymatic glycation (35, 36). Therefore, understanding how AGES transmit their signal to the nucleus will likely yield important insights into the mechanisms of these pathophysiological processes and provide targets for intervention.

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Signal Transduction by RAGE

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