Signaling Pathways for Glycated Human Serum Albumin-Induced IL-8 and MCP-1 Secretion in Human RPE Cells

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PURPOSE. To determine the signal mediators involved in glycated human serum albumin (GHSA) stimulation of interleukin (IL)-8 and monocyte chemotactic protein (MCP)-1 secretion in human retinal pigment epithelium (hRPE) cells.

METHODS. hRPE cells were stimulated by GHSA in the presence or absence of a series of kinase inhibitors. The induced IL-8 and MCP-1 mRNA and proteins were determined by reverse transcription–polymerase chain reaction (RT-PCR) and enzyme-linked immunosorbent assay (ELISA). Western blot analysis, electrophoretic mobility shift assay, and immunohistochemical staining were used to analyze activation of signaling mediators and transcription factors.

RESULTS. Incubation of hRPE cells with GHSA resulted in rapid activation of Raf-1, extracellular signal-regulated protein kinases (ERK) 1/2, p38, and the transcription factor nuclear factor (NF)-κB. Coincubation of hRPE cells with the mitogen-activated protein (MAP) kinase (MEK) inhibitor U0126; NF-κB inhibitors BAY11-7085, caffeic acid phenethyl ester (CAPE), parthenolide, and curcumin; protein kinase (PK)C inhibitor Ro518220; and protein tyrosine kinase (PTK) inhibitor genistein largely eliminated most of the stimulated production of IL-8 and MCP-1. Combined inhibition of MEK by U0126, p38 by SB202190, and Janus kinase (Jak) by AG490 revealed that GHSA stimulation of IL-8 production was predominately mediated by MEK and to a lesser extent by p38 pathways, whereas activation of MEK, p38, and Jak was required for maximal MCP-1 induction. Moreover, GHSA-stimulated IL-8 secretion was more sensitive to U0126 (50% inhibitory concentration [IC50] = 0.5 μM) than MCP-1 (IC50 = 10 μM).

CONCLUSIONS. GHSA stimulates hRPE IL-8 and MCP-1 production through divergent and overlapping, but not identical, intracellular signaling cascades. GHSA induces activation of a series of kinases including PKC, PTK, MAPK, p38, and Jak and the transcription factor NF-κB. The Raf/MAPK pathway plays an essential role in GHSA signaling. (Invest Ophthalmol Vis Sci. 2001;42:1660–1668)

Human retinal pigment epithelium (hRPE) and underlying Bruch’s membrane form the outermost layer of the blood-retina barrier. One of the major roles of hRPE cells in ocular diseases is to express various cytokines and growth factors.1,2 When stimulated, hRPE cells are programmed to release a number of chemokines, including interleukin (IL)-8 and monocyte chemotactic protein (MCP)-1.3–5 It has been shown that IL-8 is a chemoattractant, an activator for neutrophils and eosinophils, and a mediator of angiogenesis.7,8 We have previously described the increase of IL-8 in the vitreous of eyes with proliferative diabetic retinopathy.9,10 MCP-1 is capable of activating lymphocytes and monocytes, causing monocyte/macrophage infiltration in tissues.11,12 It is involved in the inflammatory response and tissue repair processes. IL-8 and MCP-1 are produced by a wide variety of human cells including hRPE cells.4,5 Various stimuli, such as IL-1β, tumor necrosis factor (TNF)-α, and glycated human serum albumin (GHSA), have been shown to stimulate hRPE IL-8 and MCP-1 secretion.3–5 GHSA is a glycation adduct. Protein glycation occurs in both normal and hyperglycemic serum when glucose nonenzymatically attaches to lysine residues of the proteins and forms labile Schiff base intermediates that undergo Amadori rearrangement and lead to the relatively stable early adducts ketamine or fructosamine.13 Depending on the protein turnover rate and ambient glucose concentration, the early glycated proteins may eventually form irreversible advanced glycation end products (AGEs). Glycated proteins are drastically increased under hyperglycemic conditions. The plasma levels of GHSA may vary from normal (400 μg/ml) to diabetic (1000 μg/ml).14

The enhanced protein glycation can be observed in experimental diabetes mellitus. A close correlation has been found between plasma glucose content and the degree of albumin glycation.14 Therefore, the amount of glycated albumin has been taken as an index of short- to intermediate-term integrated glycemic control.15 Increasing evidence has suggested that early glycated albumin is not just an index of glycemia or the precursor of AGEs. By itself, it may have important direct impacts on cellular functions and thus may play a pathophysiological role in microvascular complications of diabetic nephropathy and retinopathy.3,16–19 The pathophysiological role of early glycated albumin is further evidenced by the finding of existence of specific receptors for early glycated albumin.20–24 These receptors differentially bind Amadori-modified glycated albumin, but not AGEs, suggesting that the functional role of early glycated albumin may differ from that of AGES. For example, the early and the advanced glycation products differently affect retinal microvascular cell growth.18

Our previous studies have shown that early glycation adducts of human serum albumin upregulate chemokine gene expression and protein synthesis in hRPE and keratocytes.3,25 When exposed to GHSA, hRPE cells actively secrete IL-8 and MCP-1. The stimulated expression of IL-8 is synergized by costimulation with TNF-α.26 In addition to ocular cells, early glycated albumin has been demonstrated to be the causative factor in stimulation of transforming growth factor (TGF)-β gene expression in glomerular mesangial cells.17,27,28 Results from our and other laboratories support our hypothesis that early glycated albumin is a plasma-born factor directly involved in diabetic complications and other diseases.
The cellular signaling pathways involved in early glycated albumin-induced expression of cytokines are largely unknown. Recently, Cohen et al.\(^{17}\) have demonstrated that protein kinase C (PKC)-\(\beta\) is the mediator for early glycated albumin-stimulated expression of extracellular matrix proteins in mesangial cells. The signaling pathways for GHSA-stimulated hRPE chemokine expression have not been characterized. In this study, we use a pharmacological approach to delineate the signaling pathways for GHSA stimulation of IL-8 and MCP-1 gene expression. We found that mitogen-activated protein kinase (MAPK), also referred to as extracellular signal-regulated protein kinase (ERKs p42 and p44) is the major pathway involved in stimulation of IL-8 and MCP-1 production by GHSA in hRPE cells.

**Materials and Methods**

**Materials**

GHSA, which contains 1 to 5 moles of fructosamine per mole albumin, was purchased from Sigma (St. Louis, MO). The GHSA used in our study was made by incubation of human serum albumin (HSA) with glucose for less than a week, followed by purification to exclude residual contamination with AGEs as described by Baynes et al.\(^{29}\) No measurable AGEs in these products were determined in our laboratory.\(^{30}\) Western blot analysis with anti-glycated albumin was performed to confirm the absence of AGEs.\(^{31}\) Inhibitors used in this study are summarized in Table 1. Bovine albumin used in our studies was purchased from Sigma (St. Louis, MO). The GHSA used in our studies was purchased from Exocell (Philadelphia, PA) and anti-AGEs (Wako Chemicals, Richmond, VA) also indicated that the GHSA used in this study did not contain significant amounts of AGEs (Fig. 1). The signal transduction inhibitors used in this study are summarized in Table 1. Bovine albumin AGEs were prepared by incubation of bovine albumin under sterilized conditions, as previously described.\(^{32}\)

**Cell Isolation and Culture**

hRPE cells were isolated as previously described from donor eyes obtained from the Midwest Eye Bank within 24 hours of death.\(^{5}\) In brief, the hRPE cells were isolated from Bruch’s membrane after a 1-hour incubation with papain (5 U/ml). The hRPE cells were cultured in Dulbecco’s modified essential medium (DMEM) containing 15% fetal bovine serum, penicillin G (100 U/ml), streptomycin sulfate (100 \(\mu\)g/ml). As described in our previous studies,\(^{30}\) our preliminary experiments were run in three independent conditions, as previously described.\(^{30}\)

**Enzyme-Linked Immunosorbent Assay**

The levels of immunoreactive IL-8 and MCP-1 in the hRPE supernatants were determined by a modification of a double-ligand enzyme-linked immunosorbent assay (ELISA) method, as previously described.\(^{5}\) Briefly, 96-well microtiter plates were coated with rabbit anti-IL-8 or MCP-1 antibodies for 20 hours at 4°C. Nonspecific binding sites were blocked with 2% bovine serum albumin. Diluted supernatants from hRPE cultures (50 \(\mu\)l) were added and incubated for 1 hour. The plates were then subjected to sequential incubations with biotinylated rabbit anti-cytokine (1:1000) for 45 minutes and streptavidin-peroxidase conjugate for 30 minutes. A chromogen substrate (OPD) was added, and the plates were incubated to the desired extinction and the reaction was terminated with 3 M \(\text{H}_{2}\text{SO}_{4}\). Absorbance for each well at 490 nm was read in an ELISA reader. Standards included half-log dilution of corresponding cytokine concentrations ranging from 1 pg to 100 ng/ml in a linear fashion.

**Western Blot Analysis**

For preparation of whole-cell extract, the hRPE cells were lysed with lysis buffer containing 50 mM HEPES (pH 7.4), 1% Triton X-100, 0.15 M sodium chloride, 10% glycerol, 1.5 mM magnesium chloride, 1 mM EDTA, and a mixture of protease inhibitors. After sonication and centrifugation, total cell lysate was obtained. The nuclear extracts were made as previously described.\(^{32}\) Briefly, the hRPE cells were harvested and then resuspended in buffer A (10 mM HEPES [pH 7.9], 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol [DTT], and 1 mM phenylmethylsulfonyl fluoride [PMSF]) and incubated for 15 minutes on ice. Nonidet P-40 was added to a final concentration of 0.5%, and the mixture was vortexed vigorously for 20 seconds. After 1 minute on ice, nuclei were pelleted by centrifugation at 8000 rpm for 30 seconds. The pelleted nuclei were extracted at 4°C for 1 hour with buffer C (20 mM HEPES [pH 7.9], 400 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and 1 mM PMSF) before centrifugation at 15,000 rpm for 10 minutes at 4°C. The supernatants were used as nuclear extracts. The protein concentrations of both extracts were determined with a commercial kit (Sigma).

Western blot analysis of the cellular extracts from hRPE cells followed the manufacturer’s procedure. Briefly, samples containing 10 to 20 \(\mu\)g of proteins were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), and then were electrotransferred to nitrocellulose membranes. For signal protein detection, samples were blocked with a solution of Tris-buffered saline containing 5% dry milk and 0.05% Tween-20 (TBST) at 4°C overnight, probed with appropriate rabbit polyclonal antibodies, and washed three times in TBST. Next, the membranes were incubated with horseradish peroxidase–conjugated polyclonal anti-rabbit secondary antibody for 1 hour at room temperature and washed three additional times with TBST. The membrane was then visualized using an enhanced chemiluminescence-system.
Electrophoretic Mobility Shift Assay

The Dig Gel Shift kit (Roche Molecular Biochemicals, Indianapolis, IN) was used for detecting sequence-specific DNA-binding proteins in the gel shift assays. The probes were end-labeled with digoxigenin-11-ddUTP. The labeled DNA fragments containing the NF-kB binding site in the IL-8 promoter region (5'-GCAAATCGGTAAATTTCTCAGA-3' and 5'-TCAAGAGAAATTTCCAAGTTTG-3') were incubated with the hRPE nuclear extracts (1 µg) for 15 minutes at 37°C. The reaction contained poly(dI-dC) in the absence or presence of a 125-fold excess of unlabeled oligonucleotide for specific competition. The mixture was then transferred to an 8% polyacrylamide gel for gel electrophoresis. After electrophoretic separation, the oligonucleotide–protein complexes were transferred by electroblotting to positively charged nylon membranes. The digoxigenin-labeled DNA fragments were visualized by an enzyme immunoassay using anti-digoxigenin antibody.

Reverse Transcription–Polymerase Chain Reaction

For reverse transcription–polymerase chain reaction (RT-PCR), total RNA was extracted from confluent cultures of hRPE cells by using TRIzol reagent (Gibco, Grand Island, NY), according to the manufacturer’s procedure. The extracted RNA was quantified by absorbance at 260 nm, after which 5 µg was used to make cDNA. The cDNA synthesis reaction was set up according to the protocol of the manufacturer. One microgram of total RNA was used in each amplification reaction, human b-actin sense (5'-GTCAGAGGAAATTCCACGATTTG-3') and anti-sense (5'-AGCCTCTTTCAAAAACTTCTC-3') and NF-kB binding site (5'-GCAAATCGGTAAATTTCTCAGA-3') and anti-sense (5'-TCAAGAGAAATTTCCAAGTTTG-3') probes, and 1 µl of solution from previous reverse transcription. To ensure that an equal amount of template was used in each amplification reaction, human β-actin sense primer was used.

Immunocytochemistry

The immunocytochemical staining was performed according to manufacturer’s protocol from (ABC Kit; Vector, Burlingame, CA). Nearly confluent hRPE cells were incubated with primary rabbit polyclonal antibody to NF-kB (p65) at 4°C overnight (1:200). The cell-bound antibody complexes were then visualized by development in the substrate solution containing 3,3’-diaminobenzidine (DAB) to yield a red-brown reaction product. A dilution of normal rabbit serum containing the same concentrations of nonspecific IgG as primary antibody and anti-NF-kB/p65 to unstimulated hRPE cells served as a negative control.

Statistical Analysis

The mean chemokine concentration ± SEM was determined for each assay condition. Various assay conditions were compared using Student’s t-test, and P < 0.05 was considered to be statistically significant.

RESULTS

Involvement of MAPK, p38, and Janus Kinase (jak) Pathways in GHSA Signaling

As with our early observation, early glycated proteins such as GHSA, not nonglycated HSA, stimulated hRPE to actively secrete IL-8 and MCP-1 (Fig. 2). It has been shown that MAPK plays an important role in AGE signaling. Therefore, we first determined whether activation of ERK1/2 is also required for GHSA stimulation of IL-8 and MCP-1 production. Nearly confluent RPE cells were preincubated for 0.5 hours with U0126 (0.01–20 µM) and PD98059 (50 µM), two specific inhibitors of MAPK kinase (MEK). After preincubation, the cells were challenged with 500 µg/ml of GHSA for 24 hours. ELISA assays showed that U0126 induced a dose-dependent inhibition of both IL-8 and MCP-1 production (Figs. 2A, 2B). As with our early observation, early glycated proteins such as GHSA, not nonglycated HSA, stimulated hRPE to actively secrete IL-8 and MCP-1 (Fig. 2). It has been shown that MAPK plays an important role in AGE signaling. Involvement of MAPK, p38, and Janus Kinase (jak) Pathways in GHSA Signaling

![Figure 2](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933588/) GHSA-induced hRPE IL-8 (A, C) and MCP-1 (B, D) production. The hRPE cells were pretreated with either U0126 (0.01, 0.1, 1, 10, or 20 µM) and PD98059 (50 µM; A, B), or U0126 (20 µM), SB202190 (30 µM), AG490 (50 µM), and their combination (C, D) for 30 minutes (U0126, PD98059), 2 hours (SB202190), or 13 hours (AG490), followed by coincubation with 500 µg/ml of GHSA for 24 hours. GHSA-untreated cells were incubated with the same concentration of nonglycated HSA. The media were collected and subjected to ELISA. *P < 0.05; **P < 0.01; ***P < 0.001, compared with GHSA-stimulated control.
Involvement of p38 in GHSA signaling was further supported by Western blot analysis showing that p38 protein was phosphorylated when stimulated by GHSA (Fig. 3D). In contrast, AG-490 either alone or in combination with MEK or p38 inhibitors, did not show statistically significant inhibition of IL-8 expression. On the other hand, GHSA stimulation of MCP-1 appeared to be equally inhibited by inhibitors of MEK, p38, and jak. Treatment with U0126, SB202190, or AG-490 alone inhibited MCP-1 secretion by 56%, 41%, and 38%, respectively (Fig. 2D). Simultaneous use of these three compounds or use of U0126 plus SB202190 almost completely abolished GHSA-induced MCP-1 secretion (97% and 100%, respectively). These results suggest that GHSA stimulation of MCP-1 expression is mediated by at least three signaling pathways, ERK1/2, p38, and jak, in clear contrast to the predominant mediation of IL-8 expression by ERK1/2.

**GHSA Activation of Mitogen-Activated Protein Kinase (MAPK) through Raf-1 Pathway**

Ras and Raf are known to be the two upstream signal molecules that activate MAPK kinase MEK and then activate ERK1/2. To prove that GHSA activates the typical Raf-1/MAPK signaling pathway, hRPE cells were incubated with GHSA at concentrations from 250 to 2000 μg/ml. The cell lysates were harvested at 10 or 30 minutes after stimulation and then immunoblotted with anti-ERK1/2 or anti-phospho-specific ERK1/2 antibodies. Activation of ERK1/2 was estimated by normalizing phosphorylated ERK1/2 with phosphorylation-independent ERK1/2 on Western blot analysis. As shown in Figure 3B, phosphorylated ERK1/2 was barely detectable in unstimulated cells. After stimulation by GHSA, a time- and dose-dependent increase in ERK activation was observed (Figs. 3B, 3C). GHSA activation of ERK1/2 was observed as early as 10 minutes after stimulation. Longer incubation of the cells with GHSA for 30 minutes enhanced phosphorylation of ERK1/2 by 8.7-fold. GHSA at concentrations of 500, 1000, and 2000 μg/ml enhanced phosphorylation of ERK1/2 by 1.6-, 2.3-, and 2.5-fold compared with that obtained with 250 μg/ml GHSA. As with stimulated IL-8 production by GHSA, activation of ERK1/2 reached a plateau when GHSA concentrations were higher than 1000 μg/ml. The enhanced phosphorylation of ERK1/2 by GHSA was abolished by 20 μM of the MEK inhibitor U0126 (Fig. 3C).

Our previous studies have shown that stimulation of IL-8 secretion by GHSA is at transcription level. To correlate activation of MEK with stimulation of IL-8 production, the hRPE cells were cultured in media containing HSA, GHSA, or GHSA plus U0126 or PD98059. Figure 3A illustrates that hRPE IL-8 mRNA was induced by GHSA. U0126 and PD98059 resulted in a reduced steady state level of IL-8 mRNA that was induced by GHSA, suggesting a close correlation between activation of ERK1/2 and stimulation of IL-8 gene expression.

To determine whether activation of ERK1/2 by GHSA is through Raf, a phospho-specific Raf-1 antibody was used to measure the level of Raf-1 activation by GHSA. When hRPE cells were incubated with GHSA at 500 and 2000 μg/ml for 30 minutes, the induced phosphorylation of Raf-1 by GHSA was markedly increased (Fig. 3D), suggesting that GHSA-induced ERK1/2 activation is mediated by the Raf/MAPK signaling pathway.

**GHSA Activation of NF-κB**

NF-κB is one of the transcription factors involved in IL-8 and MCP-1 gene expression. To determine involvement of NF-κB in GHSA-stimulated hRPE IL-8 and MCP-1 expression, four approaches were used in this study. First, hRPE monolayers were stained immunohistochemically by anti-NF-κB. In un-

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**Figure 3.** RT-PCR of GHSA-induced IL-8 mRNA (A) and Western blot analysis of GHSA-induced activation of ERK1/2, p38, and Raf-1 in hRPE cells (B, C, and D). The cells were treated with 500 μg/ml of HSA or GHSA in the presence or absence of PD98059 (50 μM) and U0126 (20 μM) for 6 hours, and total mRNA was isolated. For Western blots of proteins from cell lysates, anti-ERK1/2, anti-active ERK1/2, anti-p38, anti-active-p38, and anti-active Raf-1 were used. GHSA-untreated cells were incubated with the same concentration of nonglycated HSA. The differential inhibition of IL-8 and MCP-1 secretion by 50 mg/ml of HSA or GHSA was observed when SB202190 was coincubated with the most potent MEK inhibitor U0126. This result suggests that ERK and p38 may be involved in parallel pathways leading to a common downstream mediator that is predominantly activated by MEK.
stimulated cells, NF-κB was not detected in nuclei (Fig. 4A). In contrast, nuclear translocation of NF-κB was evident after hRPE cells were treated with 500 μg/ml of GHSA (B, C) or 500 μg/ml of HSA (A) for 3 hours. Cells in (A) and (B) were stained with rabbit anti-human NF-κB antibody (1:200). Cells in (C) were stained with normal rabbit serum serving as primary antibody. GHSA-untreated cells were incubated with the same concentration of nonglycated HSA.

Figure 4. Immunohistochemical detection of NF-κB nuclear translocation in cultured hRPE cells. hRPE cells were exposed to 500 μg/ml of GHSA (B, C) or 500 μg/ml of HSA (A) for 3 hours. Cells in (A) and (B) were stained with rabbit anti-human NF-κB antibody (1:200). Cells in (C) were stained with normal rabbit serum serving as primary antibody. GHSA-untreated cells were incubated with the same concentration of nonglycated HSA.

TABLE 2. Effect of NF-κB Inhibitors on GHSA-Induced IL-8 and MCP-1 Secretion by hRPE Cells

<table>
<thead>
<tr>
<th>Stimulus/Inhibitor</th>
<th>IL-8</th>
<th>MCP-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (HSA)</td>
<td>0</td>
<td>0.86 ± 0.21</td>
</tr>
<tr>
<td>GHSA</td>
<td>28.68 ± 0.84</td>
<td>117.7 ± 3.81</td>
</tr>
<tr>
<td>GHSA/parthenolide</td>
<td>0*</td>
<td>0.27 ± 0.13*</td>
</tr>
<tr>
<td>GHSA/BAY 11-7085</td>
<td>0*</td>
<td>0.19 ± 0.11*</td>
</tr>
<tr>
<td>GHSA/CAPE</td>
<td>0*</td>
<td>0.71 ± 0.33*</td>
</tr>
</tbody>
</table>

Data are expressed in nanograms/milliliter. The hRPE cells were pretreated or untreated with parthenolide (50 μM) or BAY 11-7085 (40 μM) for 1 hour or CAPE (25 μg/ml) for 2 hours. GHSA (500 μg/ml) was then added. The media were collected 24 hours after hRPE exposure to GHSA. IL-8 and MCP-1 were determined by ELISA.

* P < 0.0001, compared with GHSA alone.

whereas NF-κB was not detectable in nuclear extracts from unstimulated cells. Because activation of the NF-κB family of transcription factors is regulated principally by phosphorylation and subsequent degradation of the inhibitory IκBα subunit, we further assessed the relative levels of IκBα in treated and untreated cells. As we expected, after exposure to GHSA (500 μg/ml), IκBα was rapidly degraded (Fig. 5B). Fourth, we measured activation of NF-κB by using an electrophoretic mobility shift assay. Binding of specific probes to the NF-κB-binding site of the promoter region in the IL-8 gene was observed 3 hours after induction with GHSA (500 μg/ml; Fig. 5C).

Moreover, to further analyze the role of NF-κB activation in GHSA signaling, curcumin was added to GHSA-stimulated cells. Curcumin has been demonstrated to block a signal leading to
activation of NF-κB–inducing kinase (NIK). Our results showed that curcumin reduced production of IL-8 and MCP-1 in a dose-dependent manner. At a 20-μM concentration, curcumin inhibited IL-8 and MCP-1 production by 68% and 51%, respectively, whereas at 50 μM, IL-8 and MCP-1 production by hRPEs was totally blocked (Fig. 6). We noted with interest that concomitantly with its inhibitory effect on IL-8 and MCP-1 production, curcumin almost completely abolished activation of ERK1/2. This suggests that curcumin may also suppress GHSA-activation of ERK through the signal upstream from NIK.

**PKC and Protein Tyrosine Kinase in GHSA-Induced Chemokine Secretion**

To determine the role of PKC and protein tyrosine kinase (PTK) in GHSA-stimulated expression of hRPE IL-8 and MCP-1, the PKC-specific inhibitors Ro318220 (10 μM) and calphostin C (125 nM) and the PTK inhibitors genistein (25 μg/ml) and herbimycin A (10 μM) were used to treat hRPE cells. Ro318220 and calphostin C reduced GHSA-stimulated IL-8 and MCP-1 secretion by 90% and 31% and by 92% and 54%, respectively (Fig. 7). Genistein and herbimycin A reduced 88% and 56% of IL-8 and 90% and 52% of MCP-1 production, respectively (Fig. 7). Because MEK inhibitors reduced the GHSA-stimulated chemokine induction, it was of interest to know whether the reduction in GHSA-induced chemokine production by PKC and PTK inhibitors is mediated through activation of Raf-1/MAPK. In this experiment, activation of ERK1/2 by GHSA was determined in the presence or absence of Ro318220 (10 μM) and genistein (25 μg/ml). As shown in Figure 7, genistein and Ro318220 did not block GHSA-induced ERK1/2 phosphorylation, suggesting that PKC and PTK act independently of ERK1/2 activation in GHSA signaling.

**DISCUSSION**

Ocular neovascularization, blood vessel leakage and leukocytic infiltration into the choroid and retina are commonly seen in a number of retinal and choroidal diseases. It has been thought that hRPE cells may play an important role in these pathophysiological processes by actively secreting a wide variety of chemoattractants, such as IL-8 and MCP-1. These chemoattractants can be induced by a variety of factors including serum-derived factors such as GHSA. Our studies have shown that GHSA is an important candidate responsible for these processes among serum-derived factors. It is generally believed that hyperglycemia is the major contributing factor leading to the development of diabetic retinopathy. The link between hyperglycemia-associated excessive glycation and development of proliferative diabetic retinopathy (PDR) has been suggested by several observations. The pathologic role of glycated protein is further evidenced by the findings that lowering glycohemoglobin through improving blood glucose control results in a beneficial impact on diabetic retinopathy. Because of retinal or choriocapillaris vascular leakage, circulating glycated serum proteins may accumulate in extracellular spaces or be locally formed after the leakage. Plasma GHSA is markedly elevated in patients with diabetes.
microcapillary permeability may further cause direct hRPE-GSHA contact.

hRPE IL-8 and MCP-1 are induced by various factors such as IL-1β, TNF-α, and GSHA and to a lesser extent by lipopolysaccharide (LPS). Either GSHA produced in vitro (this and our previous studies) or GSHA purified from plasma (Exocell; data not shown) strongly induced hRPE cells to produce IL-8 and MCP-1. To ensure that the observed stimulation was not due to contamination of IL-1β and TNF-α in the GSHA preparations or by an autocrine mechanism, a few pretests were included. Our previous studies have demonstrated that IL-1β and TNF-α- mediated IL-8 and MCP-1 secretion is sensitive to inhibition by specific neutralizing antibodies, whereas under the same conditions GSHA-induced IL-8 and MCP-1 secretion is unchanged.3 In addition, the ELISA used in this study is able to detect concentrations (10–50 pg/ml) of IL-1β and TNF-α much lower than those required to stimulate the same levels of hRPE IL-8 and MCP-1 secretion by 500 μg/ml of GSHA. These experiments thus exclude stimulation by IL-1β and TNF-α and an autocrine mechanism responsible for the observed stimulation by GSHA. We have also demonstrated substantial inhibition of LPS-induced IL-8 and MCP-1 secretion by polymyxin B. However, the same concentration of polymyxin B does not affect the induction of hRPE IL-8 and MCP-1 production by GSHA, suggesting that the observed stimulation by GSHA is not by LPS contaminant.3

Cell activation in response to AGEs is associated with increased expression of extracellular matrix proteins, vascular adhesion molecules, cytokines, and growth factors. Activation of Ras/MAPK, JAK/STAT, nitric oxide synthase, transcription factor NF-κB, and AP-1 has been linked to cell response to AGEs. Although GSHA and AGEs represent different developing stages of protein glycation, the receptors for early glycation products have been shown to be distinct from that of AGEs.20–22 In fact, AGE does not stimulate hRPE IL-8 and MCP-1 secretion (data not shown). Therefore, it was important in this study to delineate the signaling pathways for GSHA stimulation of hRPE IL-8 and MCP-1 production. As with AGEs, GSHA also activates the Raf/MAPK pathway and transcription factor NF-κB. Similar to AGE signaling, the JAK/STAT pathway is also important for GSHA-stimulated MCP-1 production. However, the JAK/STAT pathway is unlikely to be involved in GSHA stimulation of IL-8 secretion. In addition, the p38 pathway has not been linked to AGE signaling, whereas p38 is obviously important for GSHA stimulation of IL-8 and MCP-1 expression.

Among the three known transcription factors AP-1, NF-IL-6, and NF-κB for IL-8 gene expression, NF-κB represents the major transcription factor in most cases. Similarly, activation of NF-κB is required for MCP-1 gene expression in hRPE cells. Activation of NF-κB occurs in response to a wide variety of stimuli such as IL-1β, TNFα, LPS, and many other stress- or injury-related factors. NF-κB is a pleiotropic transcription factor that regulates activation of various inflammatory genes. Our results show that GSHA enhances nuclear translocation of NF-κB. Furthermore, NF-κB inhibition results in concomitant blockade of GSHA-induced hRPE IL-8 and MCP-1 gene expression.

MAPK pathways play a key role in a variety of cellular responses, such as cell proliferation, differentiation, tumor promotion, and cell death. The MAPK pathway has been shown to be important for IL-8 production in many cell types such as human monocytes and neutrophils stimulated by lipid-associated membrane protein f (LAMP), hist cells by adenocarcinoma, epithelial cells by bacterial or viral infection, endothelial cells by metals, and intercellular adhesion molecule (ICAM)-1 stimulated HL-60 cells by okadica acid and orthovanadate. The MAPK pathway has been shown not to be involved in IL-8 expression by IgG-stimulated human peripheral blood mononuclear cells. In contrast to IL-8, engagement of MAPK in MCP-1 production has not been reported, and in a few reports the MAPK pathway appears not to be required for MCP-1 production. In the present study, GSHA stimulation of IL-8 and MCP-1 production was closely correlated with activation of Raf and ERK1/2. The MEK inhibitor U1026 abolished 81% of GSHA-induced IL-8 and 73% of GSHA-induced MCP-1 production. This inhibition is concomitant with abrogation of ERK1/2 activation. These results suggest that the Raf/MAPK pathway may play an important role in GSHA signaling, leading to activation of IL-8 and MCP-1 transcription in hRPE cells.

Although Raf/MAPK activation and nuclear translocation of NF-κB may represent the major pathway for GSHA signaling in hRPE cells, the signal cascades leading to activation of IL-8 and MCP-1 expression are rather complicated. First, although activation of p38 is required for both IL-8 and MCP-1 induction by GSHA, p38 activation is more effective for inducing MCP-1 than IL-8. The relative contribution of p38 to IL-8 expression may be cell-type dependent. For example, in monocytes LPS- and osmotic stress-stimulated IL-8 expression have been shown to be entirely mediated by p38, whereas p38 is insufficient for maximal stimulation of IL-8 production in other cells. Sec- ond, jak activation appears to be involved in GSHA induction of MCP-1, but not of IL-8. Our data showed complete blockade of GSHA-stimulated MCP-1 secretion by simultaneous administration of inhibitors for ERK1/2, p38, and jak2. This JAK/STAT-dependent regulation of MCP-1 production is consistent with the previous observations in mesangial cells stimulated by leukemia inhibitory factor and in endothelial cells stimulated by IL-13. The IL-8 production induced by GSHA was insensitive to the jak2 inhibitor AG490 and was incompletely inhibited by blocking ERK1/2 and p38 (76%), suggesting that there might be a jak-independent mechanism underlying the remaining 24% production of IL-8 in these cells. One possibility is that AP-1 remains activated by GSHA through JNK/SAPK pathway. Furthermore, in addition to NF-κB and AP-1, another transcription factor for IL-8 gene expression is likely to be concomitantly activated by GSHA. This factor may be NF-IL6, because it is known to be involved in IL-8 gene expression in other cell types. Finally, Ro318220, a specific inhibitor of PKC, and genistein, a potent inhibitor of PTK, strongly inhibited GSHA stimulation of IL-8 by 90% and 88%, and inhibited GSHA stimulation of MCP-1 by 92% and 90%, respectively. However, these two compounds had essentially no effect on GSHA activation of ERK1/2, suggesting that there are PKC- and PTK-dependent pathways downstream of MAPK.

That GSHA induces IL-8 and MCP-1 production by overlapping but distinct pathways may explain our early findings that show different kinetics for GSHA-induced IL-8 and MCP-1 expression. Results in this study taken together suggest that GSHA stimulates hRPE IL-8 and MCP-1 production through divergent signaling cascades that involve activation of a series of kinases, including PKC, PTK, MAPK, p38, and jak, and activation of at least one transcription factor, NF-κB. Activation of the p42/p44 MAPK pathway may represent the major, but not the only, pathway for GSHA stimulation of IL-8 and MCP-1 expression in hRPE cells.

References


