

**BACKGROUND:** We have previously shown that incubation of human endothelial cells with mast cell granules results in potentiation of lipopolysaccharide-induced production of interleukin-6 and interleukin-8.

**Aims:** The objective of the present study was to identify candidate molecules and signal transduction pathways involved in the synergy between mast cell granules and lipopolysaccharide on endothelial cell activation.

**Methods:** Human umbilical vein endothelial cells were incubated with rat mast cell granules in the presence and absence of lipopolysaccharide, and IL-6 production was quantified. The status of c-Jun amino-terminal kinase and extracellular signal-regulated kinase 1/2 activation, nuclear factor- $\kappa$ B translocation and intracellular calcium levels were determined to identify the mechanism of synergy between mast cell granules and lipopolysaccharide.

**Results:** Mast cell granules induced low levels of interleukin-6 production by endothelial cells, and this effect was markedly enhanced by lipopolysaccharide. The results revealed that both serine proteases and histamine present in mast cell granules were involved in this activation process. Mast cell granules increased intracellular calcium, and activated c-Jun amino-terminal kinase and extracellular signal-regulated kinase 1/2. The combination of lipopolysaccharide and mast cell granules prolonged c-Jun amino-terminal kinase activity beyond the duration of induction by either stimulant alone and was entirely due to active proteases. However, both proteases and histamine contributed to calcium mobilization and extracellular signal-regulated kinase 1/2 activation. The nuclear translocation of nuclear factor- $\kappa$ B proteins was of greater magnitude in endothelial cells treated with the combination of mast cell granules and lipopolysaccharide.

**Conclusions:** Mast cell granule serine proteases and histamine can amplify lipopolysaccharide-induced endothelial cell activation, which involves calcium mobilization, mitogen-activated protein kinase activation and nuclear factor- $\kappa$ B translocation.

**Key words:** Histamine, Serine protease, Lipopolysaccharide, Endothelial cells

## Signal transduction pathways in mast cell granule-mediated endothelial cell activation

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## Introduction

Vascular endothelium functions as a selective barrier between blood and tissues, and regulates many aspects of the inflammatory and immune responses. The activation of endothelial cells, with resultant syntheses of both pro-inflammatory and anti-inflammatory mediators and the expression of adhesion molecules, is critical for immune surveillance and disease progression.<sup>1–4</sup>

Mast cells are integral constituents of the vessel wall, and their numbers are elevated in vascular diseases such as atherosclerosis.<sup>5–7</sup> When activated,

mast cells release vasoactive amines, prostaglandins, leukotrienes, cytokines, proteoglycans, and proteases that are potent modulators of endothelial cell function.<sup>5</sup> Previous reports from our laboratory have shown that incubation of human endothelial cells with rat mast cell granules (MCG) resulted in potentiation of lipopolysaccharide (LPS)-induced interleukin (IL)-6 and IL-8 production.<sup>8,9</sup> Further studies have shown that LPS-induced production of IL-6 by human umbilical vein endothelial cells (HUVECs) was amplified by protease-activated receptor (PAR) agonist peptides<sup>10</sup> and histamine.<sup>11</sup> These results indicated that at least two components of the MCG,

proteases and histamine, participate in LPS-induced synergy of endothelial cell activation. Activation of endothelial cells by extracellular stimuli involves participation of various signal transduction pathways including intracellular  $\text{Ca}^{2+}$ , mitogen-activated protein (MAP) kinase activity, and nuclear factor (NF)- $\kappa\text{B}$  translocation.<sup>12-15</sup> The purpose of this study was to evaluate the relative contribution of proteases and histamine in MCG-mediated endothelial cell activation, and to identify the signal transduction pathways that are involved in this process. The results presented in this report demonstrate that MCG are capable of inducing intracellular  $\text{Ca}^{2+}$  mobilization, activation of MAP kinases, and translocation of NF- $\kappa\text{B}$ , and that both serine proteases and histamine present in MCG participate in the potentiation of LPS-induced endothelial cell activation.

## Materials and methods

### Materials

HUVECs, endothelial cell growth medium-2 with the recommended growth factors, *N*-2-hydroxyethylpiperazine-*N'*-2-ethane-sulfonic acid, Hank's balanced salt solution, trypsin-ethylenediaminetetraacetate (EDTA), trypsin neutralizing solution, and trypsin were purchased from Clonetics (San Diego, CA, USA). Minimum Essential Medium and fetal bovine serum were purchased from Hyclone Laboratories (Logan, UT, USA). Heparin, penicillin, streptomycin, metrizamide, *Escherichia coli* LPS and the protease inhibitor cocktail were obtained from Sigma Chemical Co. (St Louis, MO, USA).  $\gamma$ -[<sup>32</sup>P]-adenosine triphosphate (ATP) was supplied by New England Nuclear (Boston, MA, USA). The enzyme-linked immunosorbent assay (ELISA) kits for IL-6 were purchased from R & D systems (Minneapolis, MN, USA). Calcium crimson and Fluo-4 were purchased from Molecular Probes (Eugene, OR, USA). The NF- $\kappa\text{B}$ -specific oligonucleotide and its complementary strand were synthesized at the Biotechnology Support Facility of the University of Kansas Medical Center. Protein A/G-coated agarose beads, glutathione-S-transferase (GST)-c-Jun, anti-goat and anti-mouse immunoglobulin G (IgG)-horseradish peroxidase, polyclonal antibodies against c-Jun amino-terminal kinase (JNK1) (C-17), extracellular signal-regulated kinase-1 (ERK1) (C-16) and ERK2 (C-14), and monoclonal IgG against phosphorylated ERK (p-ERK), which recognizes both phosphorylated ERK1 and ERK2, were obtained from Santa Cruz (Santa Cruz, CA, USA). MAP kinase kinase (MEK) inhibitor PD98059 was purchased from Calbiochem (San Diego, CA, USA). Bradford protein assay reagents were purchased from Bio-Rad (Hercules, CA, US). Nitrocellulose membrane and western blotting detection reagents were purchased from

Amersham (Piscataway, NJ, USA). All other reagents were supplied by Fisher Scientific (Pittsburgh, PA, USA).

### Culture of HUVECs

HUVECs were grown in endothelial cell growth medium-2 containing the recommended growth factors and 5% fetal bovine serum at 37°C in a humidified atmosphere containing 5%  $\text{CO}_2$ . The cells used in all experiments were between three and six passages.

### Preparation of mast cell granules

Three-month-old to four-month-old Sprague-Dawley rats (300–400 g) were the source of peritoneal mast cells. The rats were euthanized using inhaled Halothane. The methods employed for the isolation of mast cells and MCG have been described previously.<sup>8,16</sup> MCG were aliquoted and frozen at –20°C until use. The concentrations of MCG used in each experiment were expressed as the equivalent of the starting mast cell number.

### Treatment of MCG with phenylmethylsulfonyl fluoride

MCG were treated with medium or the serine protease inhibitor phenylmethylsulfonyl fluoride (PMSF) (1 mmol/l) for 12 h at 4°C, and were dialyzed against 100 mmol/l of Tris (pH 7.5) for 4 h with three changes of buffer.

### Measurement of protease activity and histamine levels in MCG

Aliquots of PMSF-treated and control MCG preparations were analyzed for their protease activity and histamine content. The serine protease activity (chymase) in MCG preparation was assayed spectrophotometrically at 405 nm by monitoring the hydrolysis of succinyl-phenylalanyl-leucyl-phenylalanyl-*p*-nitroanilide. The reaction was carried out at 37°C in 150 mmol/l of Tris (pH 7.6) in the presence of 1 mmol/l of substrate and the MCG preparation (equivalent to 0.5 million mast cells) in a final volume of 1 ml. The enzyme activity was continuously monitored and the rate of reaction was calculated using the extinction coefficient of 8800/M/cm for *p*-nitroanilide as described elsewhere.<sup>17</sup> Histamine content in mast cell granules was quantified using an immuno-competition assay at the IBT Reference Laboratory (Lenexa, KS, USA) using a commercially available ELISA kit.

### Assay of IL-6 production

HUVECs (10,000) were added to each of the wells of a 96-well microtiter plate and allowed to adhere for 24 h. Following adherence, activating stimuli or medium were added to the monolayers and the final volume adjusted to 0.2 ml. The cells were incubated for the indicated time periods at 37°C under the atmosphere of 5% humidified CO<sub>2</sub>. After the incubation, the culture supernatants were analyzed for IL-6 levels by ELISA according to the manufacturer's protocol.

### Determination of intracellular calcium mobilization

HUVECs were plated on glass cover slips and allowed to grow to confluency within 48 h. Aliquots of MCG equivalent to  $3 \times 10^5$  mast cells were added to cover slips on which monolayers of HUVECs ( $6 \times 10^5$ ) were grown. Cells were incubated with the Ca<sup>2+</sup>-sensing fluorophore, Fluo-4 (1 μmol/l), for 45 min at 37°C in 5% CO<sub>2</sub>. Cover slips were placed in a Fluorochamber on an Olympus Fluoview 300 Confocal Microscope. Cells were illuminated with an argon laser at 488 nm and emitted light was detected at 510 nm. Images were captured every 6 sec over a 10-min period. In additional experiments, the cells were imaged on an inverted Nikon microscope with a SPOT fluorescence sensing camera. In this case, cells were loaded with the Ca<sup>2+</sup> indicator calcium crimson. Background images were collected from each field and digitally subtracted from the regions of interest. Images were analyzed with the Fluoview and Photoshop software, allowing mean fluorescence values to be obtained from individual cells over time. In order to normalize for variations in dye loading, the initial three fluorescence values for each cell were averaged to determine the basal fluorescence level. This value was referred to as  $F_0$ . Each subsequent fluorescence value ( $F$ ) was divided by  $F_0$ , and the  $F/F_0$  ratio provides a clear illustration of changes in Ca<sup>2+</sup> over time. Statistical analysis of fluorescence values was completed using SigmaStat.

### Assay of JNK activity

Confluent HUVEC monolayers were treated with indicated concentrations of MCG in the presence or absence of LPS at 37°C for selected periods of time. After incubation, the cells were lysed with Triton Lysis Buffer (TLB) containing 20 mmol/l of Tris (pH 7.4), 137 mmol/l of NaCl, 10% (vol/vol) glycerol, 1% (vol/vol) Triton X-100, 2 mmol/l of EDTA, 25 mmol/l of β-glycerolphosphate, 2 mmol/l of sodium pyrophosphate, 1% (vol/vol) protease inhibitor cocktail and 0.5 mmol/l of dithiothreitol at 4°C for 30 min. Cell debris was removed by centrifugation of the

lysate at 13,000 × *g* for 10 min. After quantification of the protein content, samples were incubated with 40 μl of protein A/G-coated agarose beads and 0.6 μg of goat polyclonal antibody against JNK1 for 2 h at 4°C. The beads were washed by centrifugation and resuspended in TLB buffer and, thereafter, kinase buffer (25 mmol/l of hydroxyethylpiperazine-*N'*-2-ethane-sulfonic acid (pH 7.5), 12.5 mmol/l of MgCl<sub>2</sub>, 25 mmol/l of β-glycerolphosphate supplemented with 1 mmol/l of sodium orthovanadate and 0.5 mmol/l of dithiothreitol). Each sample was incubated with GST-c-Jun as the substrate for JNK1 in the presence of 0.1 mmol/l of unlabeled ATP, 5 μCi of γ-[<sup>32</sup>P]ATP, and kinase buffer in a volume of 20 μl for 15 min at 30°C. Thereafter, reactions were terminated by boiling in 2 × sodium dodecyl sulfate (SDS) sample buffer for 5 min. Samples were subsequently subjected to electrophoresis on 12% SDS-polyacrylamide gel and visualized by autoradiography. Protein loads were monitored in western blot using anti-JNK1 antibody.

### Assay of phosphorylated ERK1/2

Confluent HUVEC monolayers were incubated with indicated concentrations of MCG or histamine. Whenever applicable, the incubations also contained diphenhydramine and PD98059 to block H-1 receptors and MEK-1 activation, respectively. After treatment with indicated agents, the cells were lysed with TLB at 4°C for 30 min. Cell debris was removed by centrifugation at 13,000 × *g* for 10 min. After quantification of the protein content in the supernatants, aliquots normalized for protein concentrations were mixed with equal volumes of 2 × SDS sample buffer and heated to 100°C for 10 min. Samples were electrophoresed on 12% SDS-polyacrylamide gels and transferred onto a nitrocellulose filter. The filter was treated with 5% non-fat milk in phosphate-buffered saline and incubated with the antibody specific for phosphorylated ERK1/2. The reaction was detected by enhanced chemiluminescence. Protein loads were monitored by western blot using anti-ERK1/2 antibody.

### NF-κB activation and assay

To evaluate the role of NF-κB in MCG-mediated and LPS-mediated activation of endothelial cells, the nuclear NF-κB proteins were analyzed by electrophoretic mobility shift assay (EMSA). Confluent HUVEC monolayers were treated with MCG, LPS or the combination of MCG and LPS for the indicated time intervals at 37°C. Preparation of nuclear extracts was performed as described previously.<sup>18,19</sup> EMSA was carried out utilizing specific oligonucleotide containing NF-κB binding site 5'-AGTTGAGGG-GACTTTCCCAGGC-3'. The specific oligonucleotide

and its complementary strand were annealed and then end-labeled using  $\gamma$ -[ $^{32}$ P]ATP and T4-polynucleotide kinase. Nuclear protein extracts (1  $\mu$ g) were mixed with 3  $\mu$ g of poly(dI-dC).poly(dI-dC) and ~ 2 ng (100,000–400,000 cpm) of end-labeled DNA in 30  $\mu$ l of EMSA buffer and incubated for 30 min at 25°C. The EMSA buffer for NF- $\kappa$ B consisted of 10 mmol/l of Tris-HCl (pH 7.5) containing 40 mmol/l of NaCl, 1 mmol/l of EDTA, 1 mmol/l of  $\beta$ -mercaptoethanol, 4% glycerol, 0.1% NP-40 and 15  $\mu$ mol/l of bovine serum albumin. Following the initial binding reaction, 20  $\mu$ l of the mixture was electrophoresed at 150 V for 3 h at 25°C through a native 6% polyacrylamide gel, which was prepared in 45 mmol/l of Tris-borate buffer containing 1 mmol/l of EDTA. After electrophoresis, the gel was dried and processed for autoradiography.

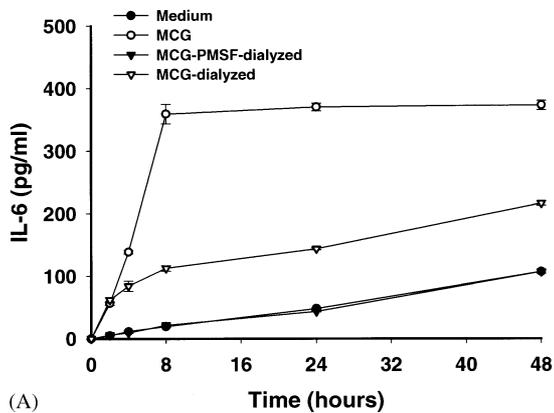
## Results

### Effect of MCG on LPS-induced IL-6 production by HUVECs

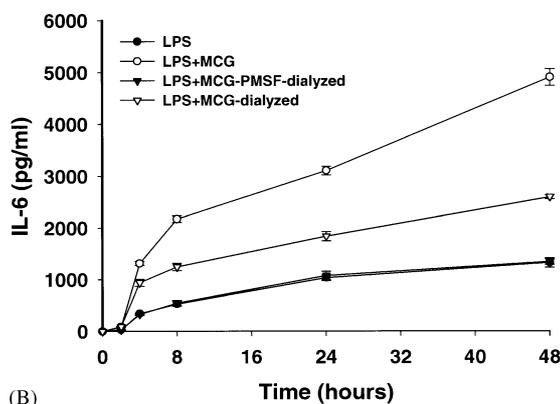
The results depicted in Fig. 1 demonstrate the effects of MCG on naïve and LPS-activated HUVECs. Production of IL-6 by HUVECs was detected as early as 2 h after incubation with MCG. IL-6 production gradually increased and plateaued by 8 h, and remained unchanged throughout the remaining 48 h. In the presence of LPS, production of IL-6 induced by MCG was significantly amplified during the 48-h incubation period. Dialysis of MCG, which removed > 99% of histamine (9158 and 0.19 ng/10<sup>6</sup> mast cell equivalent for control and dialyzed MCG, respectively) decreased endothelial cell IL-6 production by 40–60% either in the presence or absence of LPS. Inhibition of serine protease activity in MCG by treatment with PMSF (0.72 and 0 U/10<sup>6</sup> mast cell equivalent for control and PMSF-treated MCG, respectively) and depletion of histamine completely abolished the direct and modulating effect.

### Effect of MCG and LPS on Ca<sup>2+</sup> mobilization in HUVECs

The effect of MCG, LPS and the combination of MCG and LPS on Ca<sup>2+</sup> mobilization in HUVECs was studied. Transient changes in free intracellular Ca<sup>2+</sup> were measured in confluent monolayers of HUVEC using fluorescent markers. The time course and magnitude of responses for MCG, LPS and the combination are shown in Fig. 2A. The kinetics of transient changes in Ca<sup>2+</sup> was similar for all agonists with each agonist added 2 min into the experiment. The peak transient changes in Ca<sup>2+</sup> were plotted for each of the agonists as shown in Fig. 2B.  $F/F_0$  values for control cells bathed in media over 6 min were



(A)



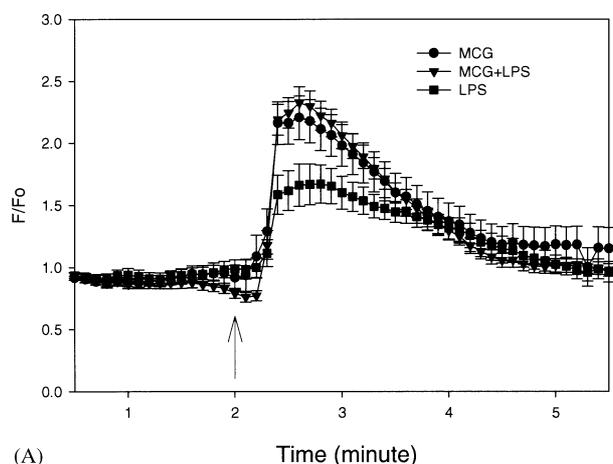
(B)

FIG. 1. Kinetics of IL-6 production by LPS-activated and MCG-activated HUVECs. HUVEC monolayers (10,000) were cultured with PMSF-treated and dialyzed MCG, dialyzed MCG or intact MCG (5000 mast cell equivalents) in the absence (A) and presence (B) of 100 ng/ml of LPS for the time intervals indicated. IL-6 concentrations in the culture media were assayed by ELISA. Values are the mean  $\pm$  standard error of the mean of quadruplicate culture wells.

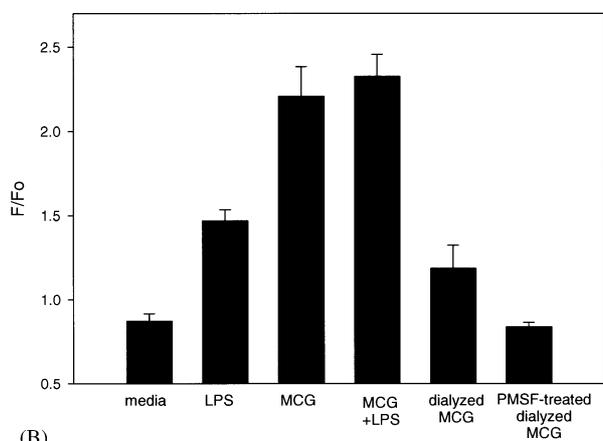
$0.87 \pm 0.04$  ( $n = 53$  cells), demonstrating the level of photobleaching that the fluorophore underwent with normal laser illumination. Exposure to LPS (100 ng/ml) induced a rapid increase in fluorescence indicating rising Ca<sup>2+</sup> levels ( $F/F_0 = 1.47 \pm 0.07$ ;  $n = 78$ ). Application of MCG caused a greater transient peaking at  $2.21 \pm 0.18$  ( $n = 66$ ). Dialysis of MCG, which removed histamine, blunted the MCG response to  $1.19 \pm 0.14$  ( $n = 24$ ). Inhibition of serine protease activity by treatment with PMSF and subsequent dialysis completely abrogated the effect of MCG ( $0.84 \pm 0.03$ ;  $n = 16$ ). The combined application of MCG and LPS caused a peak transient change that was only slightly greater than MCG alone ( $F/F_0 = 2.33 \pm 0.13$ ;  $n = 41$ ).

### Effect of MCG and LPS on JNK activation in HUVECs

Activation of JNK plays a significant role in signal transduction pathways in endothelial cells.<sup>20–22</sup> Therefore, JNK activity was evaluated after activation



(A)



(B)

FIG. 2. (A) Time course of MCG-induced and LPS-induced intracellular  $\text{Ca}^{2+}$  levels. Fluo-4-labeled HUVECs ( $6 \times 10^5$ ) were monitored with confocal microscopy during stimulation with LPS (100 ng/ml), MCG ( $3 \times 10^5$  mast cell equivalents), and a combination of both agonists (applied at the arrow). The data are plotted as  $F/F_0$  as described in the Materials and methods section. The fluorescence values (indicating free intracellular  $\text{Ca}^{2+}$ ) increased after the addition of each agonist. Each data point represents the mean  $\pm$  standard error of the mean. This is representative of three experiments. (B) MCG-induced and LPS-induced peak transient changes in intracellular  $\text{Ca}^{2+}$  levels. Peak  $\text{Ca}^{2+}$  transient changes (obtained within 2–4 min) were plotted summarizing all experiments. The data are plotted as  $F/F_0$  as described in the Materials and methods section. The results presented are from one of three similar experiments using two different fluorophores (calcium crimson and Fluo-4) on both a confocal microscope and a SPOT fluorescence microscope. In total, over 150 cells were analyzed for each condition except for dialyzed MCG. Each bar represents the mean  $\pm$  standard error of the mean.

of endothelial cells by LPS and MCG (Fig. 3). Incubation of HUVECs with MCG resulted in low level activation of JNK1 between 30 and 60 min, which returned to basal levels by 3 h. Incubation of HUVECs with LPS stimulated peak JNK activity at 3 h, which returned to baseline by 6 h (data for 1 and 4 h not shown). However, simultaneous exposure of HUVECs to LPS and MCG prolonged the activation of JNK1 for up to 8 h. The results presented in Fig. 1 and those reported previously<sup>10,11</sup> demonstrate that

both mast cell protease and histamine are involved in MCG-induced endothelial cell activation. Therefore, to further assess the role of proteases and histamine on MCG-induced JNK activation, MCG were incubated with PMSF (1 mmol/l) and then subjected to dialysis. The treatment of MCG with PMSF completely inhibited serine protease (chymase) activity, and dialysis depleted the histamine content. As depicted in Fig. 3, the depletion of histamine alone did not affect MCG's ability to induce JNK1 activation. On the other hand, inhibition of protease activity by PMSF treatment abolished the ability of MCG to prolong LPS-induced activation of JNK1.

### Effect of MCG on ERK1/2 phosphorylation in HUVECs

ERK is a recognized signaling pathway in endothelial cell activation.<sup>23,24</sup> Results presented in Fig. 4 show that MCG stimulated ERK1/2 activation in HUVECs as determined by quantification of p-ERK by western blot. Incubation of HUVECs with MCG at an endothelial cell to mast cell ratio of 2:1 resulted in marked increase in p-ERK at 10 min, which peaked at 30 min and decreased with time to the basal level within 2 h (Fig. 4A). As noted in Fig. 4B, the activation of ERK was MCG dose dependent, and plateaued at the endothelial cell to mast cell ratio of 2:1. MCG-induced ERK1/2 phosphorylation was completely blocked by MEK inhibitor PD98059<sup>25</sup> at a concentration of 30  $\mu\text{mol/l}$ , confirming the involvement of MEK activation (data not shown).

Mast cell granules depleted of both active protease and histamine failed to induce ERK1/2 activation (Fig. 5A). Our previous study has shown that the effect of histamine on endothelial cell production of proinflammatory cytokines is completely abolished by the H-1 receptor antagonist diphenhydramine, but not by the H-2 antagonist famotidine.<sup>11</sup> To further explore the direct role of histamine in MCG-induced ERK activation in HUVECs, studies were conducted with the H-1 receptor antagonist diphenhydramine. As shown in Fig. 5B, histamine-induced ERK1/2 activation was completely abrogated by diphenhydramine (25  $\mu\text{mol/l}$ ), whereas MCG-induced effects were only partially inhibited. These results indicate that both the serine protease and histamine present in MCG are capable of independently activating ERK1/2.

### Activation of NF- $\kappa$ B proteins

Promoters of the IL-6 gene contain recognition sequences for the transcription factor NF- $\kappa$ B.<sup>26,27</sup> EMSAs were carried out using nuclear extracts of HUVECs to examine the effects of MCG and LPS on NF- $\kappa$ B translocation. The exposure of HUVECs to MCG in the presence of LPS for 3 h substantially increased translocation of NF- $\kappa$ B proteins to the

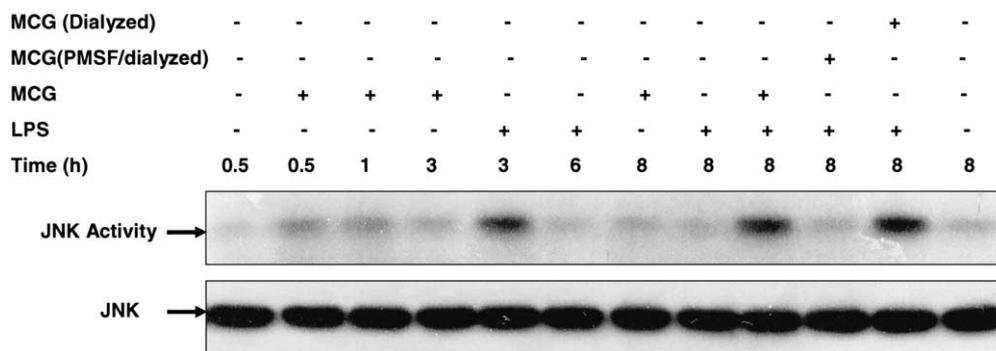


FIG. 3. Kinetics of JNK activation in HUVECs after stimulation with MCG and LPS. Confluent HUVECs ( $5 \times 10^6$ ) were stimulated with MCG, dialyzed MCG or dialyzed PMSF-treated MCG ( $2.5 \times 10^6$  mast cell equivalents) alone or in a combination with LPS. Cell lysates were prepared after the indicated time intervals. Immune complex kinase assays were performed as described in Materials and methods using GST-c-Jun as a substrate for JNK. Protein loads were monitored by western blot using anti-JNK1 antibody. Similar results were noted in two additional experiments.

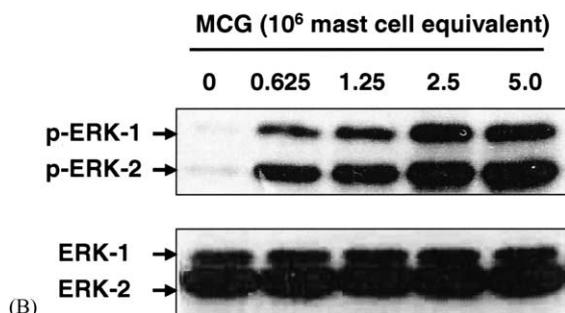
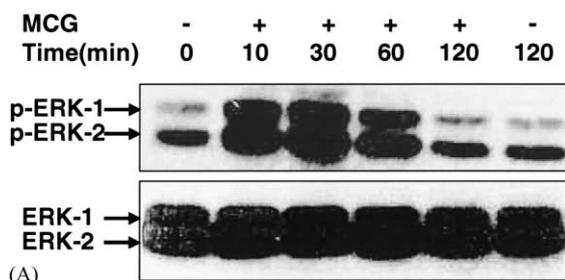


FIG. 4. The kinetics of MCG-mediated ERK1/2 activation in HUVECs. Confluent HUVECs ( $5 \times 10^6$ ) were treated with MCG ( $2.5 \times 10^6$  mast cell equivalents) for the time intervals indicated (A) or treated with the indicated doses of MCG for 30 min (B). After treatments, cell lysates were prepared and subjected to SDS-polyacrylamide gel electrophoresis followed by immunoblotting with phospho-specific ERK1/2 monoclonal antibody. Protein loads were monitored by western blot using ERK1/2 antibodies. Similar results were obtained in three experiments.

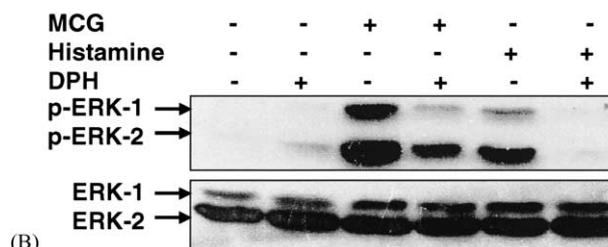
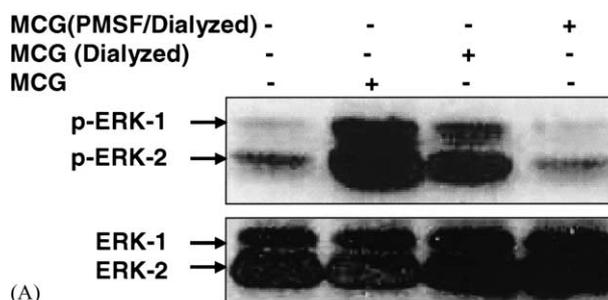
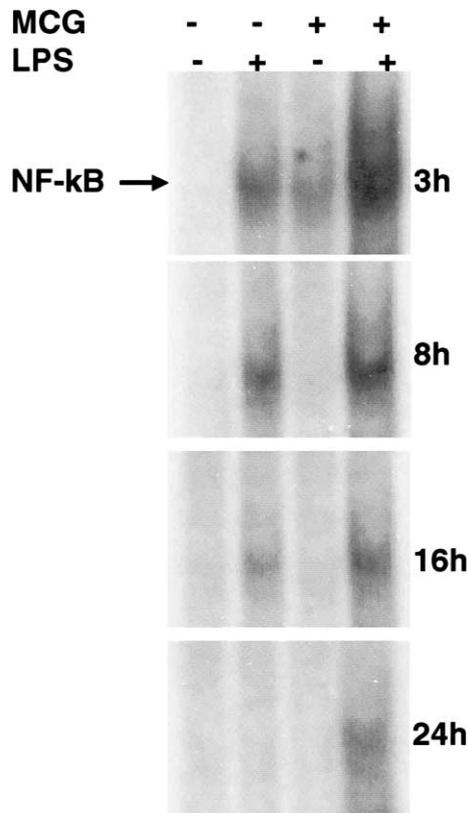


FIG. 5. The involvement of proteases and histamine in MCG-induced activation of ERK1/2. Confluent HUVECs ( $5 \times 10^6$ ) were incubated with MCG, dialyzed MCG, PMSF-pretreated and dialyzed MCG ( $2.5 \times 10^6$  mast cell equivalents) (A), or with histamine ( $25 \mu\text{mol/l}$ ) in the absence and presence of diphenhydramine ( $25 \mu\text{mol/l}$ ) (B) for 30 min. After the treatments, cell lysates were prepared and subjected to SDS-polyacrylamide gel electrophoresis followed by immunoblotting with phospho-specific ERK1/2 monoclonal antibody. Protein loads were monitored by western blot using ERK1/2 antibodies. Similar results were obtained in three experiments.

nuclei (Fig. 6). The exposure of HUVECs to MCG alone caused an increase in NF- $\kappa$ B protein levels in the nuclei, which was of lesser magnitude than that induced by LPS. Both the MCG-induced and LPS-induced NF- $\kappa$ B translocation returned to baseline by 8 and 16 h, respectively. The augmented levels of nuclear NF- $\kappa$ B proteins in HUVECs treated with the combination of MCG and LPS decreased gradually but remained elevated throughout the 24-h period.

### Discussion

Mast cells and their granules contain effector molecules that modulate functions of a variety of cells, including macrophages and endothelial cells.<sup>8,9,16,28</sup> The results presented here reveal that MCG directly activate human endothelial cells to release low levels of IL-6. The MCG-induced endothelial cell activation was greatly enhanced when HUVECs were simultaneously exposed to LPS. The effect of MCG on



**FIG. 6.** Electrophoretic mobility shift assay of nuclear NF- $\kappa$ B translocation in HUVECs treated with MCG and LPS. Confluent monolayers of HUVECs ( $5 \times 10^6$ ) were incubated with MCG ( $2.5 \times 10^6$  mast cell equivalents) in the presence or absence of LPS (100 ng/ml) at 37°C for time intervals as indicated. The nuclear proteins extracted from the cells were incubated with  $\gamma$ -[ $^{32}$ P]-labeled oligonucleotide containing NF- $\kappa$ B binding sites. The samples were subjected to electrophoresis on polyacrylamide gels, and the bands were identified by autoradiograph. The amplification of LPS-induced NF- $\kappa$ B translocation by MCG was also noted in three other experiments.

endothelial cell production of IL-6 was partially decreased when their histamine content was depleted. On the other hand, when both histamine and serine protease activity were absent, MCG failed to induce endothelial cell activation. These results are in agreement with our previous reports, which demonstrated a significant synergy between MCG and LPS on endothelial cell production of IL-6 and IL-8,<sup>8,9</sup> and that both histamine and serine proteases present in MCG participate in the induction of cytokine production.<sup>9-11</sup> The results of the present study demonstrate that the MCG serine protease-induced endothelial cell activation involves mobilization of intracellular  $\text{Ca}^{2+}$ , activation of JNK and ERK1/2, and increased translocation of NF- $\kappa$ B. Histamine independently or when present in the MCG stimulates endothelial cells through the type 1 histamine receptor by increasing intracellular  $\text{Ca}^{2+}$  and activating ERK1/2.

Mobilization of  $\text{Ca}^{2+}$  is an important event in receptor-mediated activation and involves inositol

phosphate turnover and protein kinase C activation.<sup>29</sup> Proteases, LPS and histamine are known to induce increases in intracellular  $\text{Ca}^{2+}$ .<sup>30-32</sup> Our previous studies with protease activated-receptor agonists demonstrated no correlation between increase in intracellular  $\text{Ca}^{2+}$  and the production of IL-6.<sup>10</sup> In the present study, both MCG and LPS induced comparable changes in intracellular  $\text{Ca}^{2+}$ , and a combination of these two agonists did not give an additive effect. In contrast, the combination of MCG and LPS synergized endothelial cell cytokine production.<sup>8,9</sup> The results suggest that the magnitude of  $\text{Ca}^{2+}$  mobilization does not directly relate to the level of endothelial cell cytokine production. It is apparent that both serine proteases and histamine contribute to MCG-mediated increase in intracellular  $\text{Ca}^{2+}$ .

The MAP kinases, ERK, p38 and JNK, are involved in endothelial activation.<sup>20-24</sup> The present results demonstrate that MCG-stimulated JNK1 activation in HUVECs occurs within 30-60 min of exposure. The kinetics of JNK1 activation by MCG is distinctly different to that of LPS, which peaked between 1 and 3 h (data not shown) and dissipated by 6 h. The treatment of HUVECs with a combination of LPS and MCG provided strong and sustained JNK1 activity for up to 8 h, which was not noted with either agonist alone. Pretreatment of MCG with PMSF, the serine protease inhibitor, completely abolished MCG-induced prolongation of JNK1 activity. On the other hand, MCG-induced JNK1 prolongation was unaffected by depletion of histamine from MCG. This was further supported by the finding that histamine neither directly activated nor prolonged LPS-induced JNK1 activation (data not shown). The failure by histamine to induce JNK1 activation is consistent with the lack of JNK activation by histamine in hamster *vas deferens* smooth muscle cells.<sup>33</sup> There are two plausible explanations for the sustained JNK1 activation by MCG protease. It has been demonstrated that LPS elevates the expression of PAR-2 on endothelial cells,<sup>34</sup> and that serine proteases activate PARs.<sup>31</sup> Therefore, the serine proteases in MCG may activate the newly expressed PARs on HUVECs to induce a second phase of JNK activation. Alternatively, MAP kinase phosphatase-1 (MKP-1) and MKP-2 have been implicated in the termination of JNK activity.<sup>35</sup> Since MCG are endocytosed by HUVECs,<sup>8</sup> these organelles containing proteases may inhibit MKP-1 and MKP-2, and permit the prolongation of LPS-induced JNK activation.

Incubation of HUVECs with MCG resulted in significant activation of ERK1/2 in a concentration-dependent and time-dependent manner. The effect of MCG-mediated activation of ERK1/2 was partially abrogated when histamine was depleted or by the addition of H-1 receptor antagonist diphenhydramine. When MCG preparations were pretreated with PMSF and subsequently dialyzed, ERK1/2 activation

was blocked. The lack of ERK1/2 activation by protease-free and histamine-free MCG was correlated with the failure to induce IL-6 production and synergize LPS-induced endothelial cell activation. The present result on the effect of histamine on ERK1/2 activation in endothelial cells is consistent with the report by Gudmundsdottir *et al.*<sup>36</sup> It is noteworthy that both serine proteases and histamine present in MCG are involved in the activation of ERK1/2, whereas only proteases participate in the activation of JNK. The activation of ERK1/2 by mast cell protease is in agreement with the recent finding of ERK1/2 activation by tryptase in human eosinophils.<sup>37</sup> Several reports in the literature implicate LPS-induced ERK1/2 activation in endothelial cells.<sup>38–40</sup> However, LPS did not activate ERK1/2 in our system and therefore the combined effect of MCG and LPS on ERK1/2 activity was not assessed. The lack of ERK activation by LPS may be explained by the deficiency of soluble CD14 and LPS binding proteins in the 72-h conditioned media that were used in our study to avoid serum-induced spontaneous ERK activation.

A number of studies have demonstrated the role of NF- $\kappa$ B in the regulation of IL-6 production.<sup>26,27</sup> The present study confirms that MCG, containing histamine and serine proteases, cooperate with LPS to synergize NF- $\kappa$ B translocation in HUVECs. Our previous study also demonstrated that activation of endothelial cells by PAR agonists<sup>10</sup> and histamine<sup>11</sup> resulted in NF- $\kappa$ B translocation, both of which were amplified by LPS. It is apparent that activation of at least two MAP kinase pathways, ERK1/2 and JNK1, are involved in the observed activation of endothelial cells by MCG and LPS. The distinct patterns by which MCG activates JNK1 and ERK1/2 are noteworthy in explaining the synergy between MCG mediators and LPS. Activation of JNK results in enhancement of nuclear c-Jun protein and AP-1 binding,<sup>41</sup> and augmented formation of c-Jun is known to induce endothelial cell activation.<sup>42,43</sup> Therefore, the prolonged activation of LPS-induced JNK by MCG is expected to enhance AP-1 binding to the promoters of IL-6 and IL-8. On the other hand, the direct activation of ERK1/2 by MCG will enhance c-Fos, which in turn can associate with c-Jun to form stable heterodimers<sup>44</sup> for sustained gene transcription. Thus, the amplification of LPS-induced endothelial cytokine production by MCG may be explained by the coordinated generation of c-Jun and c-Fos, and the enhanced translocation of NF- $\kappa$ B.

In summary, the present study demonstrates that MCG-mediated and LPS-mediated endothelial cell activation utilizes an increase in intracellular Ca<sup>2+</sup>, translocation of NF- $\kappa$ B, activation of JNK1 and ERK1/2, and that both serine proteases and histamine residing in MCG participate in potentiating the effects of LPS. These findings suggest that the combined effects of MCG mediators and LPS can induce

amplified endothelial cell activation. Therefore, mast cell degranulation together with bacterial infection may create a favorable environment for enhanced vascular inflammation.

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