Original Article

Overexpression of Receptor for Advanced Glycation End Products Induces Monocyte Chemoattractant Protein-1 Expression in Rat Vascular Smooth Muscle Cell Line

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Aim: The receptor for advanced glycation end-products (RAGE) has been suggested to play a pivotal role in the development of diabetic vasculopathy and atherosclerosis; however, due to its low expression, the physiological role of RAGE in vascular smooth muscle cells (VSMC) remains unknown.

Methods: Using VSMC lines stably expressing RAGE (RAGE-A10), we studied the molecular mechanism by which S100B, a RAGE ligand, induces proinflammatory gene expression.

Results: S100B induced NF- κ B activation and the expression of several proinflammatory genes (MCP-1, IL-6, ICAM-1) at mRNA and protein levels in RAGE-A10, among which MCP-1 expression was the most robust. S100B-induced MCP-1 expression was dose-dependently blocked by inhibitors of JNK (SP600125), p38 (SB203580), MEK-1 (U0126) as well as NF- κ B (Bay117085). In RAGE-A10, S100B activated JNK, MEK-1 and p38. S100B-induced MCP-1 promoter activity via NF- κ B binding sites and nuclear translocation of NF- κ B p65 subunit were blocked by SP600125, U0126, and SB203580 in RAGE-A10.

Conclusion: Our study demonstrates that S100B increased MCP-1 expression via NF- κ B and mitogen-activated protein kinase (JNK, ERK1/2, and p38) pathways in RAGE-overexpressed A10 cell lines. Thus, RAGE-A10 could be a useful cell model for studying the molecular mechanism(s) of up-regulated RAGE in the vasculature.

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Key words; RAGE, MCP-1, JNK, Vascular smooth muscle cell

Introduction

A receptor for advanced glycation end-products (RAGE) is a member of the immunoglobulin superfamily of cell-surface molecules; however, it has been shown that RAGE, beyond its classical concept, can be activated by a wide range of endogenous ligands, such as S100/calgranulins, amphoterin and amyloid fibrils¹⁾, which are released from injured or necrotic cells by interacting with RAGE to provoke innate immunity, providing a novel concept of “damage-associ-

ated molecular pattern molecules”. Thus, the RAGE-ligand axis is appreciated as a novel key factor for chronic inflammatory diseases, such as cancer, neurodegenerative disease, and atherosclerosis²⁾.

Dysfunction of vascular smooth muscle cells (VSMC) has been shown to play an important role in the pathogenesis of cardiovascular diseases^{3, 4)}. It has been shown that the low basal expression of RAGE in VSMC in normal vessels⁵⁾ is markedly upregulated at the site of vascular injury⁶⁾. In addition, several previous studies have shown that blockade of RAGE signaling protects against the development of atherosclerotic and neointimal vascular lesions in apoE-deficient mice and an arterial restenosis model^{7, 8)}; however, few studies have reported the molecular and cellular actions of RAGE in VSMC.

To address these issues, we established stable RAGE-expressing rat VSMC lines to elucidate the cel-

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lular and molecular mechanisms downstream of RAGE activation, and revealed that S100B-induced MCP-1 expression via RAGE in VSMC is mediated by several mitogen-activated protein (MAP) kinases and NF- κ B-dependent pathways.

Materials and Methods

Materials

S100B protein and Dulbecco's modified Eagle's medium (DMEM) were purchased from Sigma-Aldrich (St. Louis, MO); recombinant high mobility group box 1 (HMGB1) proteins from R & D Systems (Minneapolis, MN); Bay117085 from Biomol International (Plymouth Meeting, PA); fetal bovine serum (FBS) from Cell Culture Laboratories (Cleveland, OH); QIAzol Lysis Reagent from Qiagen (Valencia, CA); Eugene 6 from Roche (Mannheim, Germany); U0126, SB203580, SP600125 from Calbiochem (San Diego, CA); PCR primers were synthesized by Griner Bio-One (Tokyo, Japan).

The retroviral plasmid (pMX-IP) and the retrovirus packaging cell line (PLAT-A) were kindly provided by Dr. T. Kitamura (University of Tokyo)⁹. Immortalized human umbilical vein endothelial cell line (EAhy 926) was kindly provided by Dr C.J.S Edgell (University of North Carolina)¹⁰.

Cell Culture

The rat aortic smooth muscle cell line, A10, was cultured in DMEM supplemented with 10% FBS, 15 mM HEPES buffer, 100 mg/dl glutamine, 100 μ g/ml streptomycin and penicillin and incubated at 37°C in a humidified atmosphere of 95% air-5% CO₂. Cells were passaged with 0.05% trypsin/ 0.02% EDTA.

A10 Cell Lines Stably Expressing RAGE (RAGE-A10)

A coding region of human RAGE cDNA isolated from EAhy926 total RNA by RT-PCR, was subcloned into the Sal I and Not I sites of the retroviral vector, pMX-IP. The empty vector, pMX-IP, was used as a control. Replication defective retroviruses were generated by transient transfection of these constructs into PLAT-A, using FuGene 6 Reagent. A10 cells were then infected with retroviruses as described⁹. Individual colonies were positively selected and expanded in the presence of puromycin, and two RAGE-A10 clones were established.

Immunohistochemical Staining

Cells were seeded in a Lab-Tek chamber (Nalge Nunc, Rochester, NY), starved for 24 h, pretreated with or without SP600125 (5 μ M), U0126 (5 μ M),

or SB203580 (10 μ M) for 45 min, and then stimulated with S100B (10 μ g/ml) for 1 h. Cells were fixed with ice-cold 70% acetone for 20 min and then incubated with anti-NF- κ B p65 subunit antibody (sc109), (1:25; Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at room temperature. Secondary antibody (anti-rabbit IgG) conjugated to Alexa fluor 488 (1:2000; BD Pharmingen, San Diego, CA) was used to visualize primary antibody distribution. Laser scanning confocal microscopy was performed using a Carl Zeiss LSM 510 system (Carl Zeiss, Jena, Germany).

Immunoblotting

Immunoblot analysis was performed as described¹¹. Briefly, cells were harvested and lysed with ice-cold RIPA buffer (20 mM NaPO₄ [pH7.4], 100 mM NaCl, 1% IGEPAL-CA340, 0.25% deoxycolate, 0.1% sodium dodecyl sulfate (SDS), 30 μ g/ml aprotinin, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), and 2 mmol/L EDTA. Cell lysates (25 μ g protein) were separated by 10% SDS-PAGE. After transferring to nitrocellulose membranes, the blot was incubated with antibodies against RAGE (sc5563) (1:600; Santa Cruz Biotechnology), actin (A2066) (1:1000; Sigma-Aldrich, St. Louis, MO), ICAM-1 (sc-1511) (1:1000; Santa Cruz Biotechnology), phospho-p38 (#9215s) (1:1000; Cell Signaling Technology, Beverly, MA) and p38 (#9212) (1:1000; Cell Signaling Technology), followed by a secondary antibody conjugated with horseradish peroxidase (1:30,000; GE Healthcare, Buckinghamshire, UK) and developed with the ECL Plus detection system (GE Healthcare).

Quantification of mRNA by Real-Time RT-PCR

Total RNA extraction and first-strand cDNA synthesis were performed as described¹². The sequences of PCR primer pairs were as follows: monocyte chemoattractant protein-1 (MCP-1) forward, 5'- ATGCAG-GTCTCTGTACGCT -3', and reverse 5'- GGT-GCTGAAGTCCTTAGGGT-3' (product size, 345 bp); intercellular adhesion molecule-1 (ICAM-1) forward, 5'- GACCCTGGAGATGGAGAAGAC -3', and reverse 5'- CTTGTCCAGGTGAGGACCATA -3' (product size, 343 bp); interleukin-6 (IL-6) forward, 5'- AATCTGCTCTGGTCTTCTGGAG -3', and reverse 5'- GTTGGATGGTCTTGGTCCTTAG -3' (product size, 239 bp); matrix metalloproteinase-9 (MMP-9) forward, 5'- GACAAGAAGTGGGGTTT CTGTC -3', and reverse 5'- TAGAGCCACGAC-CATACAGATG -3' (product size, 199 bp); 18s ribosomal RNA forward, 5'- GACACGGACAGGATT-GACAG -3', and reverse 5'- AGACAAATCGCTC-CACCAAC -3' (product size, 90 bp). Steady-state

mRNA levels of rat MCP-1, ICAM-1, IL-6 and 18s ribosomal RNA were quantified with real-time quantitative RT-PCR using fluorescent SYBR Green technology (Light Cycler; Roche) as described¹³. The quantification and calculation of mRNA levels of the target sequence were performed using 18s ribosomal RNA as an endogenous internal control¹⁴.

Enzyme-Linked Immunosorbent Assay (ELISA) for MCP-1 and IL-6

Cells were seeded at a density of 1×10^5 cells/well on 12-well culture plates, starved for 24 h and then treated with or without S100B (10 μ g/ml). After 24 h incubation, MCP-1, IL-6 protein levels in the conditioned medium were determined using commercially available ELISA kits (Invitrogen, Carlsbad, CA).

Protein Kinase Assay

SAPK/JNK and ERK1/2 kinase activities were measured by commercially available assay kits (Cell Signaling Technology) as described¹⁵. Briefly, 250 μ g cell lysate was incubated with glutathione-S transferase-c-Jun (1-89) or Elk-1 fusion protein beads at 4°C overnight. Complexes were collected, washed, and then incubated with 50 μ l kinase buffer containing 100 μ M ATP at 30°C for 30 min. The reaction was terminated with 5x Lammeli sample buffer and subject to SDS-PAGE. Western blotting using phospho-c-JUN or phosphor-Elk-1 antibody was performed.

Transient Transfection and Luciferase Assay

The rat MCP-1 promoter/enhancer-driven reporter construct (pGL3-ENH), composed of a proximal promoter region and distal enhancer region containing two distinct NF- κ B binding sites (A1, A2), and dual mutation at A1 and A2 (PGL3- $\Delta\kappa$ B ENH) was used¹⁶. Cells were seeded at 1×10^5 cells/well on 24-well culture plates. Transient transfections of 0.8 μ g MCP-1 reporter constructs and 0.2 μ g thymidine kinase promoter-driven Renilla luciferase plasmids (pRL-TK) (Promega, Madison, WI) were performed using FuGene 6 reagent as described^{16, 17}. After 12 h incubation, cells were starved for 24 h and then treated with or without S100B (10 μ g/ml) for 6 h. Luciferase activities were measured by the Dual Luciferase Reporter Assay System (Promega) using Micro Lumat Plus (EG&G Berthold, Wildbad, Germany)¹⁶.

Statistical Analysis

Data are presented as the mean $\pm$ SEM and were analyzed using the unpaired Student's *t*-test or ANOVA with Dunn's post-hoc test if appropriate. $p < 0.05$ was considered significant. Graphpad Prism Version

Fig. 1. Vascular smooth muscle cell lines stably expressing RAGE

(A) Immunoblotting of RAGE (upper panel) and actin (lower panel) in A10, pMX-A10, RAGE-A10 (a, b) and rat lung; Molecular weight markers (kDa) are shown on the right. (B) Immunohistochemical staining using NF- κ B p65 subunit antibody in control cells (pMX-A10) and RAGE-A10 cells before (left panel) and after (right panel) stimulation with S100B.

5.0 (Graphpad Software, San Diego, CA) was used for statistical analysis.

Results

Stably Expressing Human RAGE-A10 Cell Lines (RAGE-A10)

Since RAGE expression was undetectable in the primary culture of rat aortic VSMC and the A10 cell line by immunoblot analysis, the A10 cell line stably expressing human RAGE cDNA encoding full-length RAGE transcript^{18, 19} was established by a bicistronic retrovirus system carrying a puromycin-resistance gene. Western blot analysis revealed that two cell lines (RAGE-A10a and -A10b) had a distinct band corresponding to the molecular size (55-kDa) of endogenous RAGE of the rat lung, whereas RAGE expression in the control cell line transfected with empty retroviral vector (pMX-A10) or in the parent A10 was barely detectable (**Fig. 1A**).

Fig. 2. S100B-induced inflammatory gene expression in RAGE-A10

RAGE-A10 and pMX-A10 cells were incubated with S100B (10 μ g/ml) (closed column) or without (open column) for 3 h; mRNA levels of (A) MCP-1, (B) ICAM-1 and (C) IL-6 were measured by real-time RT-PCR. Each column represents the mean of three independent experiments and is plotted as the fold increase over controls without S100B. Bar shows SE. * $P < 0.05$

Immunohistochemical analysis revealed that S100B, a RAGE ligand, induced the translocation of p65 subunit of NF- κ B from the cytosol to nucleus in RAGE-A10 (**Fig. 1B**, lower panel), but not in pMX-A10 (**Fig. 1B**, upper panel). These data suggest that RAGE expressed by RAGE-A10 is functional and capable of activating the NF- κ B pathway²⁰.

Proinflammatory Gene Expression in RAGE-A10

We next examined whether RAGE in RAGE-A10 is involved in proinflammatory gene expression, as measured by real-time RT-PCR. As shown in **Fig. 2**, S100B significantly ($P < 0.05$) increased mRNA levels of MCP-1, ICAM-1, and IL-6 in two cell lines, RAGE-A10a and -A10b, but not in the control cell line (pMX-A10), in which MCP-1 gene was most robustly induced (30-50-fold).

We also examined the effect of HMGB1, another RAGE ligand, on MCP-1 expression in RAGE-A10 at various concentrations. RAGE-A10 barely responds to HMGB-1: only HMGB-1 at a very high concen-

tration (1.0 μ g/ml) was capable of increasing MCP-1 mRNA expression (**Supplemental Fig. 1**).

S100B Induced Proinflammatory Gene Expression in a Time- and Dose-Dependent Fashion

S100B time-dependently (1.5-12 hr) and dose-dependently (1-10 μ g/ml) increased MCP-1, ICAM-1 and IL-6 mRNA levels (**Fig. 3A, B**). Likewise, S100B significantly ($P < 0.05$) increased the secretion of MCP-1 and IL-6 protein and the expression of ICAM-1 protein in RAGE-A10, but not in pMX-A10 (**Fig. 3C**).

S100B Induced MCP-1 Expression Via MAP Kinase- and NF- κ B-Dependent Pathways

To determine whether the NF- κ B and/or MAP kinase pathways are involved in S100B-induced MCP-1 expression in RAGE-A10, we examined the effects of various inhibitors of NF- κ B and several protein kinases (**Fig. 4**). Bay117085, an inhibitor of NF- κ B, dose-dependently (1-5 μ M) inhibited S100B-induced

Fig. 3. S100B induces MCP-1, ICAM-1, and IL-6 mRNA and protein expression in RAGE-A10

RAGE-A10 cells were incubated with S100B (10 $\mu\text{g/ml}$) (A) for the indicated times and (B) at the indicated concentrations for 4.5 h to measure MCP-1, ICAM-1 and IL-6 mRNA by real-time RT-PCR, and (C) RAGE-A10 and pMX-A10 cells were incubated with S100B (10 $\mu\text{g/ml}$) for 24 h to measure MCP-1, IL-6 protein by ELISA and ICAM-1 protein by immunoblotting. The mean of three independent experiments was calculated and plotted as described in Fig. 2; * $P < 0.05$ vs. cells without treatment.

Fig. 4. Effects of various inhibitors of protein kinases and NF- κ B on S100B-induced MCP-1 production in RAGE-A10

After pretreatment with or without (A) Bay117085, a NF- κ B inhibitor; (B) SP600125, a JNK inhibitor; (C) U0126, a MEK-1 inhibitor; and (D) SB203580, a p38 inhibitor at various doses, RAGE-A10 cells were stimulated with (closed column) and without (open column) S100B (10 $\mu\text{g/ml}$) for 24 h to measure MCP-1 protein by ELISA. Data were calculated and plotted as in Fig. 2. Bar shows SE. * $P < 0.05$ vs. cells without treatment. † $P < 0.05$ vs. cells stimulated with S100B without pretreatment.

Fig. 5. JNK activation is involved in S100B-induced MCP-1 transcriptional activation through NF- κ B binding sites in RAGE-A10

(A) RAGE-A10 cells were incubated with S100B (10 μ g/ml) for 30 min, and JNK and ERK1/2 activities and protein levels of phospho-p38 were measured. Each column represents the mean of three independent experiments and is plotted as the fold increase over controls without S100B. Bar shows SE. * P <0.05.

(B) After pretreatment with or without SP600125 (5 μ M), U0126 (5 μ M), and SB203580 (10 μ M) for 1 h, cells cotransfected with pRL-TK and pGL3-ENH or pGL3- Δ κ B ENH were stimulated with (closed column) or without (open column) S100B for 6 h; luciferase activity was then measured. Relative luciferase activities expressed as the fold increase over the control are the mean of four independent experiments. Bar shows SE. * P <0.05 vs. cells without treatment. † P <0.05 vs. cells stimulated with S100B without pretreatment.

MCP-1 expression, while SP600125 (1-5 μ M), a JNK inhibitor; SB203580 (5-10 μ M), a p38 inhibitor; and U0126 (1-5 μ M), a MEK-1 inhibitor similarly blocked S100B-induced MCP-1 expression in a dose-dependent fashion: these inhibitors did not affect MCP-1 expression in cells without S100B stimulation (**Fig. 4**). These data suggest that both NF- κ B and MAP kinase (ERK1/2, JNK, p38) pathways are involved in S100B-induced MCP-1 expression.

We further examined the involvement of MAP kinases in RAGE-dependent MCP-1 expression in RAGE-A10. S100B significantly (P <0.05) activated JNK, ERK1/2 and p38 (**Fig. 5A**) in RAGE-A10. We also found that S100B activated SEK-1 and MMK3/6, respective upstream kinases of JNK and p38, in RAGE-A10 (**Suppl. Fig. 2**). By the luciferase reporter assay using MCP-1 promoter¹⁶, S100B significantly (P <0.05) increased the promoter activity of pGL3-ENH, but not pGL3- Δ κ B ENH, whose effect was inhibited

by pretreatment with SP600125 (5 μ M), U0126 (5 μ M) and SB203580 (10 μ M) (**Fig. 5B**). Furthermore, SP600125 (5 μ M), U0126 (5 μ M) and SB203580 (10 μ M) blocked S100B-induced translocation of the p65 subunit of NF- κ B from the cytosol to nucleus in RAGE-A10 by immunohistochemical analysis (**Fig. 6**). Taken together, these findings indicate that ERK, JNK and p38 pathways are activated by S100B, and each MAP kinase pathway is involved in NF- κ B-dependent MCP-1 expression in RAGE-A10.

Discussion

In the present study using rat VSMC lines stably expressing human RAGE cDNA (RAGE-A10), we have clearly demonstrated that a RAGE ligand (S100B) induced several proinflammatory gene expressions, such as MCP-1, IL-6 and ICAM-1, among which MCP-1 mRNA and protein expression were the most

Fig. 6. Effects of inhibitors of JNK, MEK-1, and p38 on S100B-induced nuclear translocation of NF- κ B p65 subunit in RAGE-A10

Immunohistochemical staining using NF- κ B p65 subunit antibody in RAGE-A10 cells. After pretreatment without (B) or with SP600125 (5 μ M) (C), U0126 (5 μ M) (D), and SB203580 (10 μ M) (E) for 45 min, cells were stimulated with S100B (10 μ g/ml) for 1 h. Untreated cells (A) served as a control.

robust. Furthermore, the present study showed that activation of MAP kinases (ERK1/2, p38, JNK) and NF- κ B is essential for S100B-induced MCP-1 expression in RAGE-A10.

It has been shown that RAGE was upregulated at the site of vascular injury, such as neointimal lesions⁷⁾ and atherosclerotic plaque in apoE-deficient mice⁸⁾ and diabetic patients⁶⁾. Blockade of RAGE signaling by soluble RAGE and gene targeting has been shown to prevent the development and/or progression of such vascular lesions; however, few studies have reported the molecular and cellular mechanisms of RAGE action in VSMCs. Recently, it has been reported that Src tyrosine kinase activated by RAGE is involved in a series of cellular responses in porcine aortic VSMC, including activation of MAP kinases and STAT3 as well as NF- κ B-dependent proinflammatory gene expression²¹⁾. Very recently, it has been shown that RAGE activation is involved in the activation of the TGF- β -ROCK1 pathway in mouse aortic VSMC²²⁾. In contrast, several previous studies have

shown that cultured VSMC barely expressing RAGE were unable to respond to RAGE ligands^{5, 23)}. We also failed to detect RAGE expression and responses to S100B in our parent A10 cells and primary cultured rat VSMCs (unpublished observation). Such contradictory findings could be partly accounted for by the different species and experimental conditions. Nevertheless, several clinical and experimental animal studies have shown that RAGE expression is barely detectable or at a low level in blood vessels under normal conditions, but upregulated at the site of vascular injury^{6, 8, 24)}. These observations prompted us to establish a cell model mimicking RAGE-upregulated VSMC.

The molecular size of RAGE protein expressed in RAGE-A10 appears to be similar to that of the rat lung where RAGE is ubiquitously expressed. The present study further demonstrated that S100B, a RAGE ligand, activated the NF- κ B pathway by nuclear translocation of p65, a NF- κ B component. Thus, RAGE-A10 could be a useful cell model for investigating the cellular and molecular mechanism by which RAGE

exerts its effects on VSMC. Consistent with the previous study using porcine VSMC²¹⁾, S100B induced the expression of a series of NF- κ B-driven proinflammatory genes (MCP-1, ICAM-1, IL-6) in RAGE-A10 at both mRNA and protein levels. Notably, S100B robustly upregulated MCP-1 mRNA and protein expressions. Since MCP-1, a pivotal chemokine, is involved in the development of atherosclerotic plaques and neointimal formation^{25, 26)} and several previous studies have shown RAGE-dependent MCP-1 expression in VSMC^{21, 27)}, RAGE-mediated MCP-1 upregulation in VSMC may contribute to the development and/or progression of such vascular lesions.

Although previous reports have shown that many biological effects of HMGB-1 were mediated by RAGE^{28, 29)}, several recent studies have clarified that purified HMGB-1 alone is not sufficient for stimulating a RAGE-mediated response³⁰⁻³²⁾ and the interactions of additional components, such as cytokines, LPS, DNA fragment, and/or interaction with Toll-like receptors, are prerequisites for its biological effects³³⁻³⁵⁾. Thus, the present finding that RAGE-A10 barely responded to HMGB-1 may lend support to the contention that HMGB-1-mediated biological effects require not only RAGE but also additional factors.

In the present study, S100B-induced MCP-1 production in RAGE-A10 was blocked by an NF- κ B inhibitor (Bay11074), suggesting that NF- κ B activation is indispensable for RAGE-mediated MCP-1 expression. The exact signaling pathways of NF- κ B activation downstream of RAGE have not been fully understood yet. Our study indicates that S100B activated JNK, ERK1/2 and p38, and inhibitors of either of the MAP kinase pathways blocked S100B-induced nuclear translocation of the p65 subunit of NF- κ B and subsequent MCP-1 production. Furthermore, the present luciferase assay using rat MCP-1 promoter containing two NF- κ B binding sites revealed that JNK, ERK1/2, and p38 activations are involved in S100B-induced NF- κ B-dependent MCP-1 promoter activation. These results collectively suggest that RAGE-mediated MAP kinase activation acts as an upstream signal of NF- κ B activation to induce MCP-1 expression in RAGE-A10. Blockade of S100B-induced MCP-1 expression by inhibitors of MEK1 and p38, as shown in this study, is consistent with previous studies showing that both ERK1/2 and p38 are involved in RAGE-mediated NF- κ B activation and subsequent MCP-1 expression in VSMC^{21, 36)}. Although several previous studies have shown that JNK is one of the signaling pathways downstream of RAGE in endothelial cells⁸⁾ and adventitia fibroblasts³⁷⁾, the present study, to the best of our knowledge, demon-

strated for the first time the role of JNK in the RAGE-mediated response in VSMC. These results suggest that JNK as well as ERK1/2 and p38 plays a pivotal role in the development and/or progression of RAGE-induced vascular lesions.

As a limitation of this study, we adopted the A10 cell line in the present study, since the usage of primary VSMC culture is not suitable for establishing a stable RAGE-expressing cell line. Although the A10 cell line was derived from the thoracic aorta of embryonic rats showing combined characteristics between neonatal and neointimal VSMC^{38, 39)} and has been used as a common cellular model of VSMC, we could not completely reject the possibility that the cellular response of A10 may not be identical to that of *in vivo* VSMC.

In conclusion, using rat VSMC lines stably expressing human RAGE (RAGE-A10), the present study clearly demonstrated that a RAGE ligand (S100B) stimulates NF- κ B activation to induce the expression of several proinflammatory genes, among which MCP-1 expression was the most robust, and that several MAP kinases (ERK1/2, p38, JNK), are involved in S100B-induced MCP-1 expression. Thus, RAGE-A10 could be a useful cell model for investigating the molecular mechanisms of upregulated RAGE in the vasculature.

Acknowledgments

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Supplemental Fig. 1. HMGB-1 barely induced MCP-1 mRNA expression in RAGE-A10

RAGE-A10 cells were incubated with (closed column) recombinant HMGB-1 at the indicated concentrations or without (open column) for 3 h. Intracellular MCP-1 mRNA was determined by real-time RT-PCR. Each column represents the mean of three independent experiments and is plotted as the fold increase over controls without recombinant HMGB-1. Bar shows SE. * $P < 0.05$

Supplemental Fig. 2. S100B induces phosphorylation of SEK-1 and MKK3/6 in RAGE-A10

RAGE-A10 cells were incubated with S100B for 0-15 min. Equal amount of cell lysates at each time point were subjected to immunoblotting with anti-phospho-SEK-1, anti-SEK-1 (a), anti-phospho-MMK3/6 and anti-MMK3/6 (b) antibodies, respectively.