Regulation of Inducible Nitric Oxide Synthase Expression in Advanced Glycation End Product–Stimulated RAW 264.7 Cells

The Role of Heme Oxygenase-1 and Endogenous Nitric Oxide

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Advanced glycation end products (AGEs) are closely linked to the development of diabetic atherosclerosis. The current study examines the induction of inducible nitric oxide (NO) synthase (iNOS) and heme oxygenase (HO)-1 expression by AGEs, as well as the signaling pathways involved and the interplay between these two enzymes. The stimulation of RAW 264.7 cells with 6.64 or 33.2 μg/ml AGEs leads to HO-1 protein expression, iNOS protein expression, and nitrite accumulation. AGEs lead to the phosphorylation of p42/44 and p38 mitogen-activated protein kinase (MAPK). The inhibition of p42/44 MAPK and protein kinase C prevented, whereas inhibition of p38 MAPK augmented, AGE-induced nitrite release and iNOS expression. In contrast, HO-1 expression was downregulated by inhibition of p38 MAPK. Furthermore, the expression of both proteins was prevented by coincubation with acetovanillone (NADPH oxidase inhibitor). AGE-induced iNOS expression was negatively regulated by stimulation of HO-1 expression with cadmium chloride or endogenous NO. Tin-protoporphyrin IX (HO-1 inhibitor) partially reversed the cadmium chloride–mediated downregulation of iNOS expression. The current study demonstrates that multiple signaling molecules are involved in AGE-stimulated iNOS and HO-1 expression. There also exists a downregulation of iNOS by its own product as well as the products of HO-1. *Diabetes* 53:1841–1850, 2004

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AGE, advanced glycation end product; apo, apolipoprotein; DMEM, Dulbecco's modified Eagle's medium; ELISA, enzyme-linked immunosorbent assay; HO, heme oxygenase; IFN, interferon; IL, interleukin; iNOS, inducible nitric oxide synthase; L-NAME, N^{ω}-nitro-L-arginine methyl ester; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor-κB; NIH, National Institutes of Health; PKC, protein kinase C; RAGE, receptor for AGEs; Tin-PP, Tin-protoporphyrin IX.

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Advanced glycation end products (AGEs) are produced nonenzymatically by the Maillard reaction between amino acid (lysine and arginine) side chains in proteins and reducing sugars such as glucose (1). AGEs are found in the tissues of diabetic patients (2), and this accumulation has been implicated in the acceleration of diabetic microangiopathy, exemplified by retinopathy and nephropathy.

AGE-specific binding proteins were first identified in macrophages (OST-48 and the protein kinase C [PKC] substrate 80-KH) (3). In addition, two binding proteins for AGEs were identified on the surface of endothelial cells, receptor for AGEs (RAGE) and lactoferrin-like polypeptide (homologous to lactoferrin) (4). The interaction between AGEs and their receptors plays a key role in the progression of diabetic atherosclerosis and glomerulopathy (5,6).

AGEs have been shown to stimulate inducible nitric oxide (NO) synthase (iNOS) expression in endothelial cells (7), mouse macrophages (8), and RAW 264.7 cells (9,10). The high concentrations of NO produced by iNOS may play a role in the development of atherosclerosis (11). Interestingly, administration of exogenous NO donor agents causes inhibition of interferon (IFN)-γ plus interleukin (IL)-1β–induced iNOS expression in astroglial cells (12). Moreover, the inhibition of iNOS activity by N^{ω}-methyl-L-arginine or N^{ω}-nitro-L-arginine methyl ester (L-NAME) augments lipopolysaccharide (LPS) plus IFN-γ–induced iNOS expression in RAW 264.7 cells (13), suggesting that NO exerts a negative feedback effect.

Heme oxygenase (HO) is expressed in atherosclerotic lesions in both endothelial cells and foam cells (14). HO-1 is a stress-induced protein that is expressed in response to a variety of stimuli (15). HO-1 expression is implicated in protection against atherosclerosis. For example, mice deficient in both HO-1 and apolipoprotein (apo)E develop atherosclerosis more rapidly than mice deficient in apoE alone (16), and HO-1 overexpression inhibits atherosclerosis development in apoE-deficient mice (17). Furthermore, exogenous CO enhances the expression of the anti-inflammatory cytokine IL-10 through the p38 mitogen-activated protein kinase (MAPK) pathway (18). In addi-

tion, a recent study showed that HO-1 expression is upregulated in monocytes prepared from type 2 diabetic patients and is accompanied by the expression of the NADPH oxidase membrane component p22^{phox} (19). Recently, AGEs also have been shown to stimulate HO-1 expression in endothelial cells (20).

HO-1 expression also appears to be involved in the regulation of iNOS expression. For example, HO-1 induction with heme or 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 caused inhibition of iNOS expression. These effects are proposed to be mediated by products of HO-1 because iNOS downregulation is diminished in the presence of Tin-protoporphyrin IX (Tin-PP) or zinc-protoporphyrin IX (21,22), both of which are inhibitors of HO-1.

Acetovanillone is a widely used NADPH oxidase inhibitor, which works by prevention of p47^{phox} and p67^{phox} membrane translocation, which is necessary for the activation of NADPH oxidase (23). The membrane translocation of the two components is regulated through the phosphorylation of cytosolic components through the p42/44 and p38 MAPKs and PKC (24–26). NADPH oxidase is activated by incubation with diabetic erythrocytes prepared from type 1 diabetic patients and with N^{ϵ}-(carboxymethyl)lysine (27). AGEs activate NADPH oxidase, and this may lead to the production of reactive oxygen species, which can stimulate vascular cell adhesion molecule-1 expression (27) and inflammatory gene expression through nuclear factor-κB (NF-κB) activation (28).

Although it has been reported that AGEs stimulate iNOS expression, the effect of AGEs on HO-1 in macrophages has not been investigated. In addition, the relationship between AGEs, NADPH oxidase, iNOS-derived NO and HO-1 remains to be clarified. In this report, we demonstrate that AGEs stimulate iNOS and HO-1 expression. iNOS expression is stimulated by p42/44 MAPK and PKC, and this expression is modulated by HO-1 through p38 MAPK and by endogenous iNOS-derived NO.

RESEARCH DESIGN AND METHODS

Acetovanillone, BSA, D-glucose, and cadmium chloride were purchased from Sigma-Aldrich. PD 98059, SB 203580, and Calphostin C were purchased from Calbiochem (La Jolla, CA). Tin-PP was purchased from Alexis (Lausen, Switzerland). Dulbecco's modified Eagle's medium (DMEM) and penicillin-streptomycin were purchased from Invitrogen (Carlsbad, CA). All other chemicals used in this study were purchased from Fisher and were analytical reagent grade.

Cell culture. RAW 264.7 cells were obtained from American *Type Culture* Collection (Manassas, VA). Cells were cultured at 37°C in a humidified atmosphere of 5% CO_2 using DMEM containing 10% FCS, penicillin (100 units/ml), and streptomycin (100 μg/ml). In measuring nitrite concentrations, 5×10^5 RAW 264.7 cells were seeded in 12-well plates. In performing Western blots, 2×10^6 RAW 264.7 cells were seeded in 6-well plates.

Preparation of BSA and AGEs. AGEs were prepared according to previously described methods (29). Briefly, BSA and AGEs were prepared by incubation of 3.14 mg/ml BSA in the presence or absence of 200 mmol/l D-glucose and 0.5 mmol/l sodium azide in PBS (pH 7.4) at 37°C for 12 weeks with constant agitation. After incubation, BSA and AGEs were dialyzed against 6 l PBS for 24 h to remove unbound sugars, and then these preparations were passed over a polymixin column (Pierce, Rockford, IL) to minimize endotoxin contamination. The protein concentration was measured by *Dc* protein assay (Bio-Rad, Hercules, CA). All glassware was treated with E-TOXA-CLEAN (Sigma, St. Louis, MO) to remove endotoxin. AGE content was estimated by fluorescence intensity (excitation 360 nm, emission 400 nm; excitation 390 nm, emission 460 nm; and excitation 370 nm, emission 440 nm). In addition, AGEs were oxidized by periodate, leading to the production of formaldehyde, which was measured as the fluorescent 3,5-diacetyl-1,4-dihydrolutidine with acetylacetone and ammonium acetate (30). In this study, BSA and AGEs were expressed as protein concentration.

ELISA procedure. Noncompetitive and competitive enzyme-linked immunosorbent assay (ELISA) were performed as previously described (31) using the anti-AGE conjugated horseradish peroxidase antibody (Research Diagnostics, Flanders, NJ).

Nitrite measurement. Nitrite accumulation was determined by mixing equal volumes of cell culture medium and Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride, and 2% H_3PO_4). Absorbance (A_{590}) was measured using a microplate reader; standard curves were constructed with known concentrations of $NaNO_2$ (32).

Western blot analysis. To determine protein expression, 20 μg protein was separated by 7.5 or 10% SDS-polyacrylamide gel electrophoresis. Gels were transferred to a polyvinylidine fluoride membrane (for monoclonal antibody) or a nitrocellulose membrane (for polyclonal antibody) and then placed in blocking solution (Tris-buffered saline [10 mmol/l Tris, pH 8.0, and 150 mmol/l NaCl], 0.05% Tween 20, and 5% nonfat milk) for 1 h. Blots were incubated for 1 h with anti-iNOS monoclonal antibody (BD Transduction Laboratories, Franklin, NJ), anti–HO-1 polyclonal antibody (Stressgen, Canada), anti–phospho-p42/44 MAPK, p42/44 MAPK, phospho-p38 MAPK, p38 MAPK polyclonal antibodies (Cell Signaling Technology, Beverly, MA), and anti-actin monoclonal antibody (Chemicon, Temecula, CA) washed with Tris-buffered saline and 0.05% Tween 20 and incubated with horseradish peroxidase–conjugated adequate secondary antibody. Bound IgG was visualized using an enhanced chemiluminescence detection system (Pierce, IL) according to the manufacturer's protocol. Band intensities were quantified with National Institutes of Health (NIH) Image software.

iNOS activity. iNOS activity was measured by monitoring the conversion of [3H]arginine to [3H]citrulline using the NOS Assay Kit (Cayman Chemical, Ann Arbor, MI) according to the manufacturer's protocol.

RT-PCR. Total RNA was isolated using TRIzol reagents (Invitrogen). To avoid DNA contamination, 50 μg total RNA was treated with deoxyribonuclease I (TaKaRa Bio, Otsu, Japan). One microgram of deoxyribonuclease I–treated total RNA was used for RT-PCR with the One Step RNA PCR kit (TaKaRa Bio). Primers for detection of mouse RAGE mRNA were designed as follows: sense primer 5′-AACATCACAGCCCGGATTGG-3′ and anti-sense primer 5′-ACAATT TCTGGCTTCCCAGG-3′. The 304 bp is predicted for amplification of mouse RAGE mRNA. To show the absence of DNA contamination, we examined RT-PCR in the presence or absence of reverse transcriptase. The amplified products were resolved by 1.5% agarose gel electrophoresis.

Statistical analysis. Data were obtained from three (Western blots, iNOS activity) or five (nitrite measurements) separate experiments. Each value represents the mean ± SE. Statistical significance was assessed by the Student's t test, and differences between treatment groups were considered significant at $P < 0.05$.

RESULTS

Estimation of AGE concentration. To estimate the concentration of AGEs, we measured and compared the fluorescence intensity of our AGE sample to that of untreated BSA. AGEs showed 5.08-fold (excitation 360 nm, emission 400 nm), 3.91-fold (excitation 390 nm, emission 460 nm) and 5.19-fold (excitation 370 nm, emission 440 nm) higher fluorescence intensity compared with BSA. In addition, the production of formaldehyde in the AGE sample in the presence of periodate was 191.85 nmol/mg protein, whereas that in the BSA sample was 6.75 nmol/mg protein. In the absence of periodate, AGEs produced formaldehyde at a concentration of only 6.06 nmol/mg protein, whereas BSA produced 5.98 nmol/mg protein. In the noncompetitive ELISA against anti-AGE antibody, AGE samples showed an increase in absorbance at 450 nm in a concentration-dependent manner, whereas BSA controls did not show an increase. In addition, in the competitive ELISA against commercially available AGEs, addition of commercial AGEs competitively inhibited the reaction between AGE samples and anti-AGE antibody (data not shown).

AGEs stimulate NO release and iNOS expression in RAW 264.7 cells. To examine whether AGEs induced NO release in RAW 264.7 cells, we treated cells with 1.33, 6.64, and 33.2 μg/ml BSA or AGEs for 36 h and measured nitrite

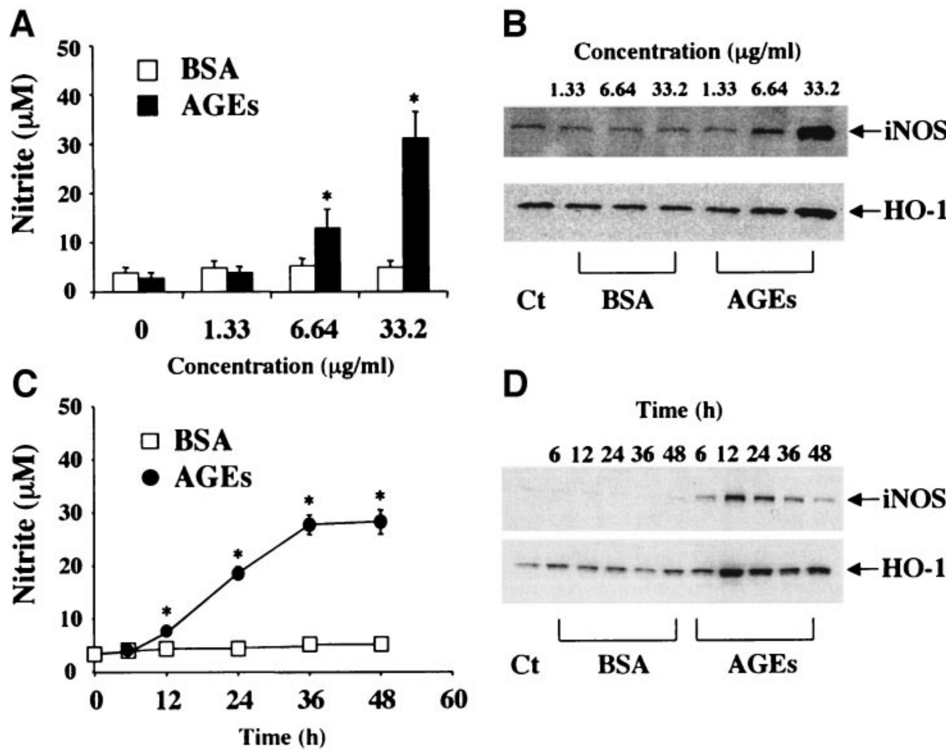

FIG. 1. Nitrite accumulation and iNOS and HO-1 protein expression in AGE-stimulated RAW 264.7 cells. Cells were incubated with BSA or AGEs (1.33–33.2 μg/ml) for 36 h (*A*), 12 h (*B*), or 0–48 h (33.2 μg/ml; *C* and *D*). Nitrite accumulation was determined by Griess assay (*A* and *C*). iNOS and HO-1 protein expression was examined by Western blot (*B* and *D*). Ct, control.

accumulation in the cell culture medium by the Griess assay (Fig. 1*A*). AGEs at 6.64 and 33.2 μg/ml led to a significant accumulation of nitrite compared with BSA treatment. Figure 1*B* shows that AGE-induced iNOS protein expression at 12 h in a concentration-dependent manner. The accumulation of nitrite after AGE (33.2 μg/ml) treatment increased to a plateau at 36 h (Fig. 1*C*). iNOS protein was transiently induced by AGEs (33.2 μg/ml), with the expression peaking at 12 h (Fig. 1D). After 24, 36, and 48 h, iNOS expression declined but was still significantly increased compared with BSA. Thus, AGEs induce iNOS expression and lead to nitrite accumulation in RAW 264.7 cells.

AGEs stimulate HO-1 expression in RAW 264.7 cells. We examined the induction of HO-1 protein expression by AGEs. Figure 1*B* shows that 1.33, 6.64, and 33.2 μg/ml AGEs induced HO-1 protein expression at 12 h in a concentration-dependent manner, and AGE (33.2 μg/ml)-induced HO-1 expression was highest at 12 h. HO-1 expression remained above baseline up to 48 h after stimulation (Fig. 1*D*).

p42/44, p38, and PKC pathways are involved in AGE-induced nitrite release, iNOS activity, and iNOS and HO-1 expression. To investigate the signaling mechanism whereby AGEs induce iNOS and HO-1 expression, we examined the activation and phosphorylation of p42/44 and p38 MAPK. p42/44 and p38 MAPK were both phosphorylated in response to AGE (33.2 μg/ml) stimulation, with a maximal activation occurring after 30 min (Fig. 2*A* and *B*).

As shown in Fig. 3*A*, we measured AGE (33.2 μg/ml)-

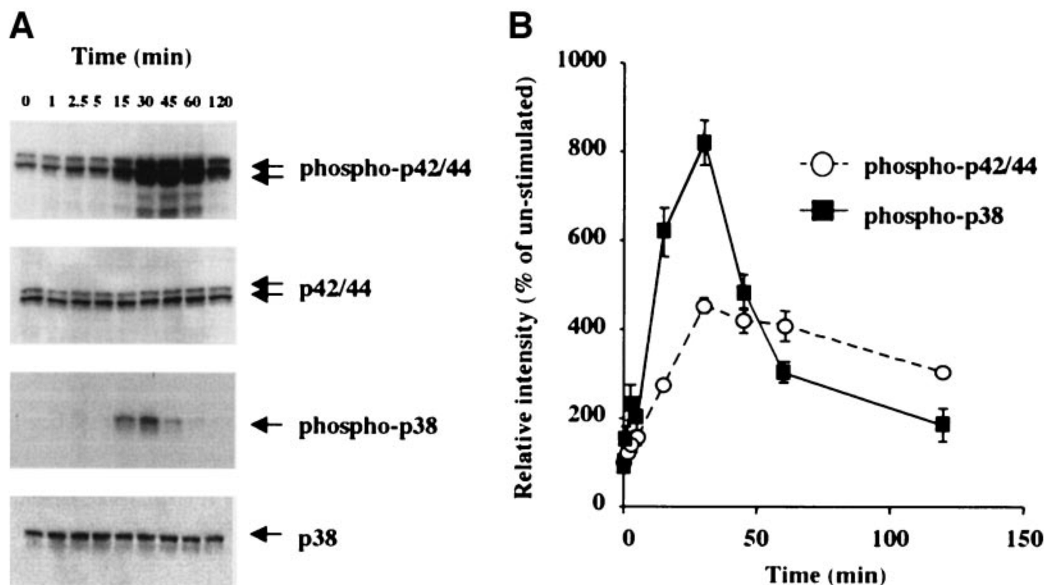

FIG. 2. Phosphorylation of p42/44 and p38 MAPK in AGE-stimulated RAW 264.7 cells. *A*: Cells were incubated with AGEs (33.2 μg/ml) for the indicated times and Western blots performed for phosphorylated and unphosphorylated p42/44 and p38 MAPK. *B*: Band intensities were quantified using NIH Image software. Phosphorylated band intensities were normalized to unphosphorylated band intensities. Data are presented as percentage of phosphorylation in unstimulated cells.

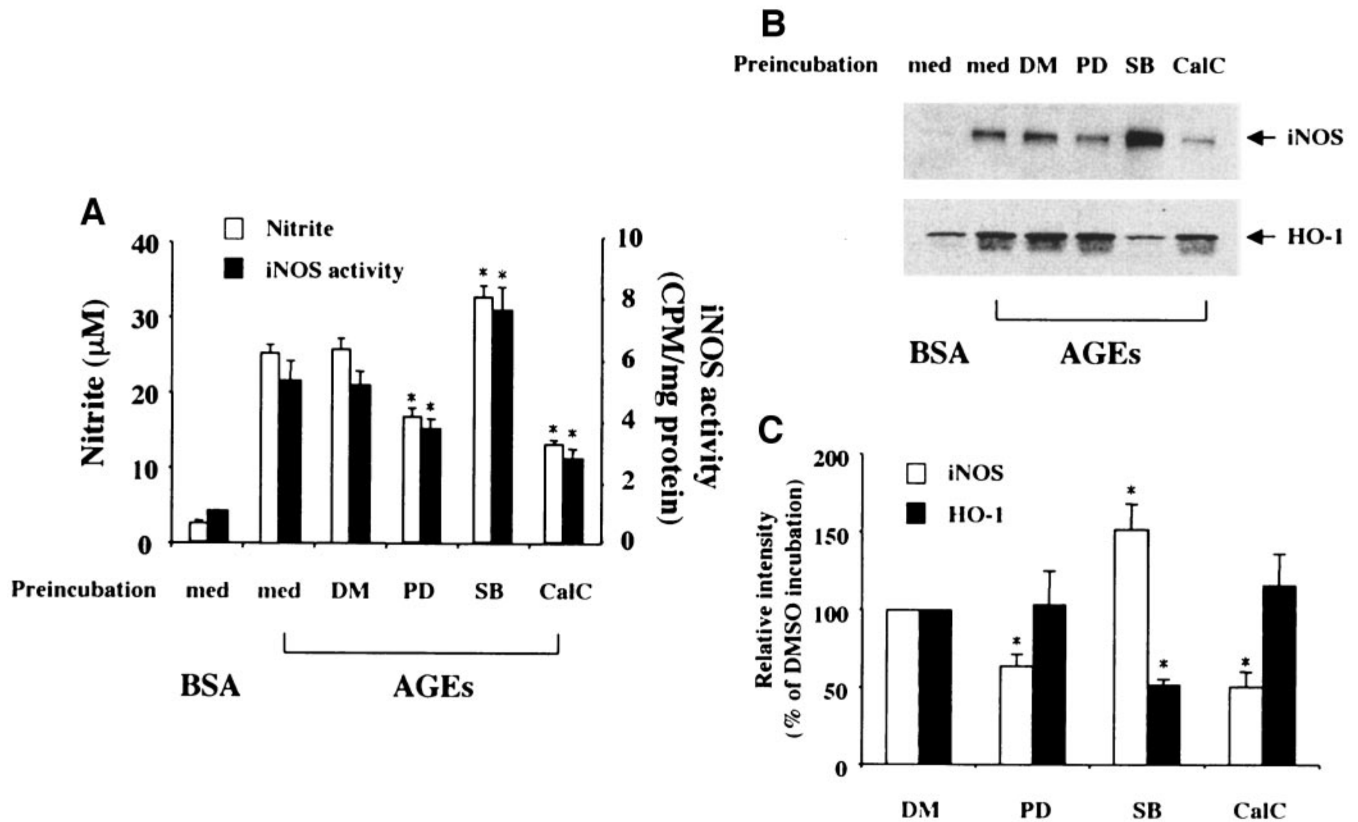

FIG. 3. Nitrite accumulation, iNOS activity, and iNOS and HO-1 protein expression in the presence of p42/44, p38 MAPK, and PKC inhibitors. Cells were preincubated for 30 min with PD 98059 (PD; 40 μmol/l), SB 203580 (SB; 40 μmol/l), calphostin C (CalC; 200 nmol/l), 0.1% DMSO (DM; vehicle), or DMEM. Washed cells were incubated with AGEs (33.2 μg/ml) for 36 h (*A*, nitrite), 24 h (*A*, iNOS activity), and 12 h (*B* and *C*). Griess reaction, iNOS activity, and Western blots were performed. Band intensities were quantified using NIH Image software. Data are presented as percentage of protein expression with DMSO preincubation (*C*). *P < 0.05 vs. DMSO vehicle.

induced nitrite release after preincubation with the p42/44 MAPK inhibitor PD 98059 (40 μmol/l), the p38 MAPK inhibitor SB 203580 (40 μmol/l), or the PKC inhibitor calphostin C (200 nmol/l). AGE-induced nitrite release was significantly inhibited by preincubation with the p42/44 MAPK inhibitor and PKC inhibitor, whereas the p38 MAPK inhibitor augmented nitrite release (Fig. 3*A*). A similar pattern was observed for iNOS activity (Fig. 3*A*) and expression (Fig. 3*B* and *C*). AGE-induced HO-1 expression was not affected by preincubation with the p42/44 MAPK or PKC inhibitor but was downregulated by preincubation with the p38 MAPK inhibitor (Fig. 3*B* and *C*). To address the possibility that DMSO affected AGE-induced nitrite release and iNOS activity and expression, we compared nitrite accumulation and iNOS activity and expression in preincubation with DMSO or DMEM. DMSO had no significant effect on any of these phenomena (Fig. 3*A*, *B*, and *C*). These data show that the induction of iNOS expression, activity, and nitrite release are upregulated by activation of the p42/44 MAPK and PKC pathways, whereas activation of p38 MAPK suppresses AGE-induced iNOS expression and potentiates HO-1 expression.

To investigate the correlation between p42/44 MAPK and PKC during the induction of iNOS expression by AGEs, we examined AGE (33.2 μg/ml)-induced p42/44 phosphorylation in the presence of calphostin C (200 nmol/l). PKC inhibition did not inhibit p42/44 MAPK phosphorylation, suggesting that PKC is not upstream of p42/44 MAPK (data not shown).

NADPH oxidase is involved in AGE-induced iNOS and HO-1 expression. To determine whether NADPH oxidase plays a role in AGE-induced iNOS and HO-1 expression, we treated the NADPH oxidase inhibitor acetovanillone (0.25, 0.5, 1, and 2 mmol/l). As shown in Fig. 4, both iNOS and HO-1 expression were decreased by coincubation with AGEs (33.2 μg/ml) and acetovanillone in a concentration-dependent manner. Nitrite release was also inhibited in the presence of acetovanillone (Fig. 5*A*).

To determine whether acetovanillone leads to the changes iNOS and HO-1 expression by inhibiting the phosphorylation of p42/44 and p38 MAPK, we examined the phosphorylation of p42/44 and p38 MAPK in the presence of 1 mmol/l acetovanillone. As shown in Fig. 5*B* and *C*, acetovanillone did not affect the AGE (33.2 μg/ml)-induced phosphorylation of p42/44 and p38 MAPK, suggesting that NADPH oxidase–mediated effects on iNOS and HO-1 expression are downstream of p42/44 and p38 MAPK phosphorylation.

RAGE is involved in AGE-induced iNOS and HO-1 expression. To investigate whether AGE-induced iNOS and HO-1 expression is mediated by RAGE, we first confirmed the expression of RAGE mRNA in RAW 264.7 cells (Fig. 6*A*). Preincubation with anti-RAGE antibody (100 μg/ml) showed a downregulation of AGE (33.2 μg/ml)-induced iNOS expression and a slight downregulation of HO-1 expression compared with normal IgG preincubation (Fig. 6*B*).

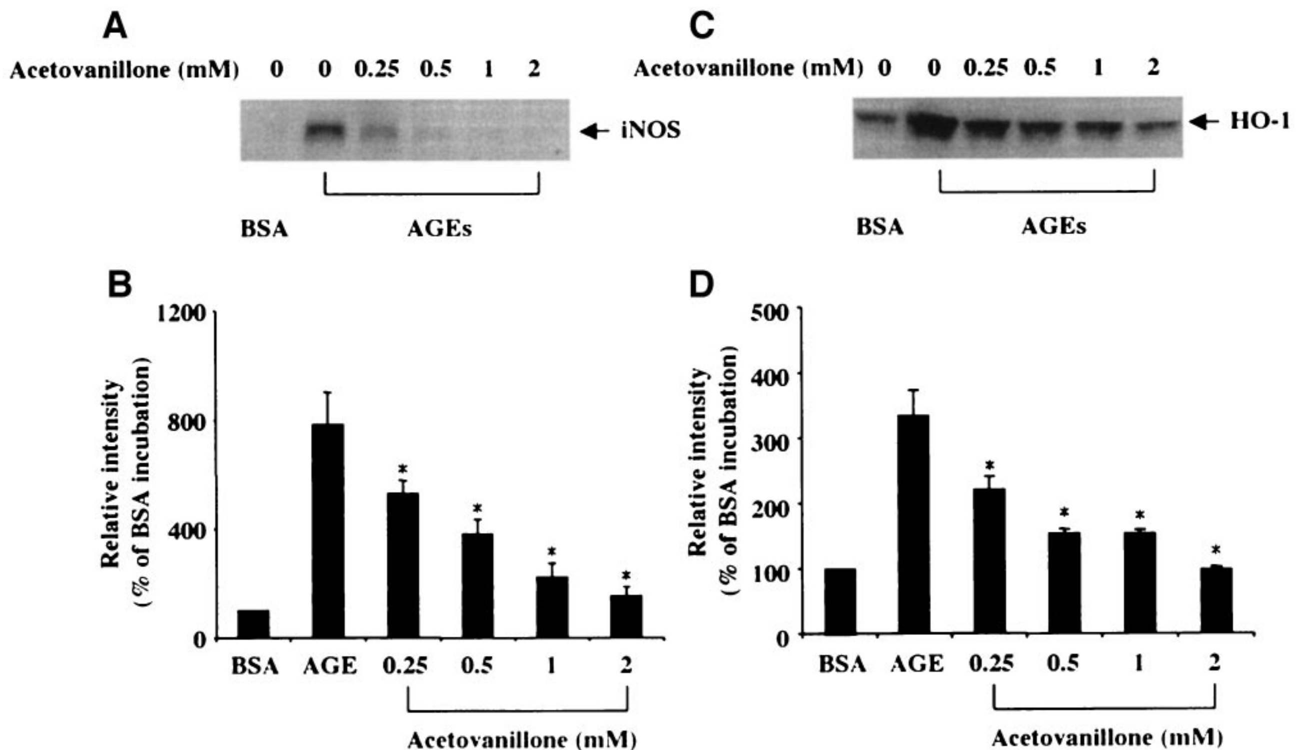

FIG. 4. AGE-induced iNOS and HO-1 protein expression in the presence of the NADPH oxidase inhibitor acetovanillone. Cells were incubated with BSA, AGEs (33.2 μg/ml), or AGEs (33.2 μg/ml) plus acetovanillone (0.25–2.0 mmol/l) for 12 h. BSA and AGEs alone were incubated in the presence of 0.1% ethanol (vehicle). Western blots were performed (*A* and *C*) and band intensities were quantified using NIH Image software. Data are presented as percentage of protein expression with BSA (*B* and *D*). $^*P < 0.05$ vs AGEs alone.

Endogenous iNOS-derived NO inhibits AGE-induced iNOS expression. To examine whether endogenous NO regulates AGE-induced iNOS expression, we coincubated AGEs (33.2 μg/ml) and the NO synthase inhibitor L-NAME (1 mmol/l). L-NAME coincubation inhibited AGE-induced nitrite release but enhanced AGE-induced iNOS expression (Fig. 7). This enhancement of iNOS expression was apparent at 24 and 36 h poststimulation (Fig. 7*B*). A similar experiment was performed to determine the effect of endogenous NO on HO-1 expression. L-NAME did not affect AGE-stimulated HO-1 expression over 36 h. Therefore, after AGE activation, NO may feed back to downregulate iNOS and reduce NO production, without affecting HO-1 expression.

HO-1 downregulates AGE-induced iNOS expression. NO appears to regulate iNOS expression. The effects of the products of HO-1 on iNOS expression and activity were also investigated. We preincubated RAW 264.7 cells for 3 h with cadmium chloride (25 μmol/l), leading to the induction of HO-1 expression, and after further treatment with AGEs (33.2 μg/ml), examined NO production and iNOS expression. HO-1 expression was increased by cadmium chloride preincubation. Cadmium chloride preincubation led to an inhibition of AGE-induced nitrite release (Fig. 8*A*) and iNOS expression (Fig. 8*B*). Cadmium chloride alone did not induce iNOS expression (Fig. 8*B*). To examine whether iNOS downregulation in the presence of cadmium chloride is mediated by HO-1 induction, we used Tin-PP to inhibit HO-1 activity. As shown in Fig. 8*C* and *D*, Tin-PP (50 μmol/l) partially reversed cadmium chloride–induced iNOS downregulation.

DISCUSSION

AGEs are formed from the reaction between proteins and glucose. This reaction, termed the Maillard reaction, leads to formation of products that are brown colored and fluorescent (1). Among the many reported AGEs, HbA_{1c} (produced from the reaction of Hb β-chain and glucose) is used as a marker for diabetes (33). Accumulation of AGEs is observed in diabetic patients (2) and is known as an important factor in the development of diabetic atherosclerosis, nephropathy, and glomerulopathy. In studies using apoE-deficient mice injected with streptozotocin, accelerated diabetic atherosclerosis is suppressed by RAGE administration to inhibit the interaction between AGEs and endogenous RAGE (5). Diabetic nephropathy is developed in RAGE-overexpressed mice (34). Also, it has been reported that diabetic glomerulopathy is accelerated in gelactin-3–deficient mice, suggesting that the interaction between AGEs and gelactin-3 blocks the negative effects of AGEs (6). In vitro studies suggest that AGEs activate signaling pathways similar to those induced by inflammatory cytokines. For example, AGEs stimulate NADPH oxidase activity, leading to the induction of vascular adhesion molecule-1 expression and generation of tissue factor in endothelial cells (27). This vascular adhesion molecule-1 induction is mediated by NF-κB. Indeed, NF-κB activation is observed with N^{ϵ}-(carboxymethyl)l-ysine (35) and glyceraldehyde- and glycoaldehyde-derived AGEs (36) in endothelial cells.

iNOS expression is upregulated by stimulation with LPS, IFN-γ, tumor necrosis factor-α, IL-1β, as well as AGEs in endothelial cells (7) and mouse macrophages (8). Re-

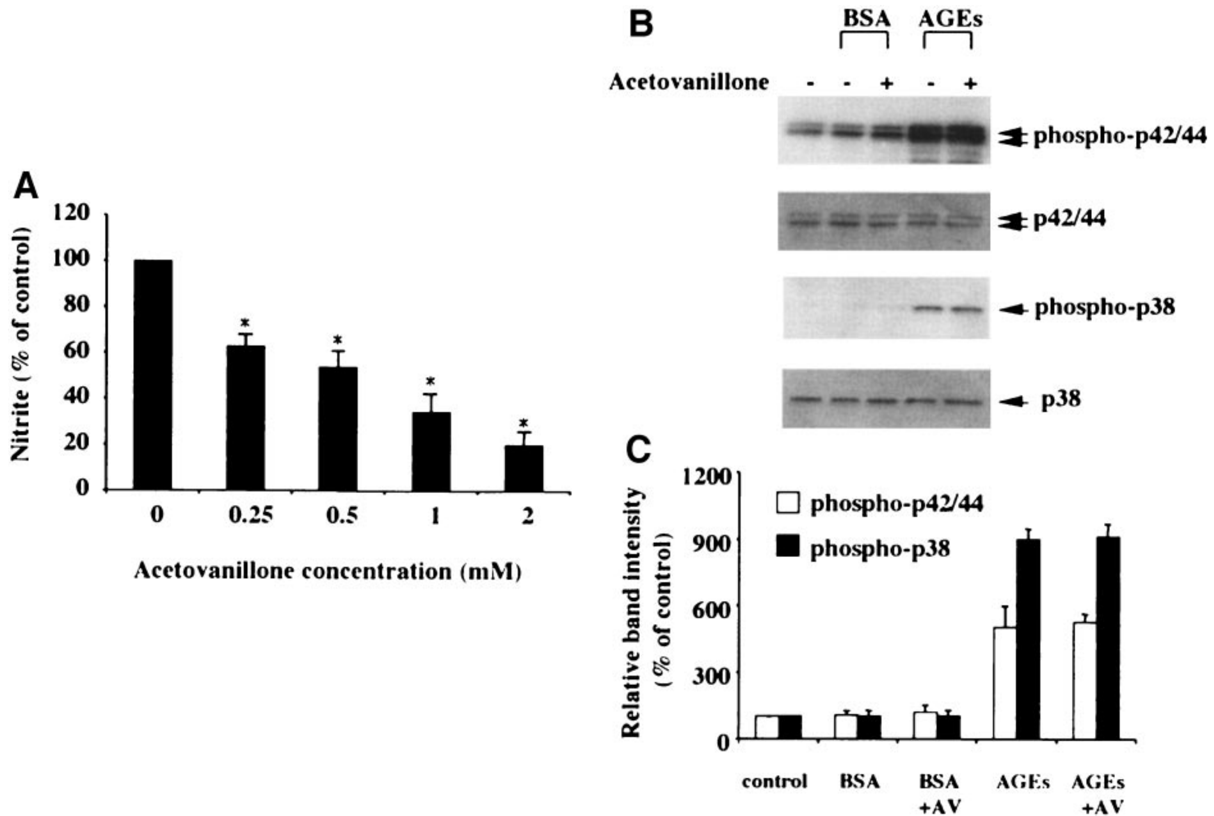

FIG. 5. AGE-stimulated nitrite release and phosphorylation of p42/44 and p38 MAPK in the presence of the NADPH oxidase inhibitor acetovanillone (AV). ***A*****: Cells were incubated with BSA, AGEs (33.2 μg/ml), or AGEs (33.2 μg/ml) plus acetovanillone (0.25–2 mmol/l) for 36 h and nitrite measurement performed. AGEs were incubated in the presence of 0.1% ethanol (vehicle). Data represent nitrite accumulation as percentage of control. *P < 0.05 vs the absence of acetovanillone.** ***B*****: Cells were incubated with BSA or AGEs (33.2 μg/ml) in the presence or absence of acetovanillone (1 mmol/l) for 30 min, and Western blots were performed.** ***C*****: Band intensities were quantified using NIH Image software. Phosphorylated band intensities were normalized to unphosphorylated band intensities. Data are presented as percentage of phosphorylation in control cells.**

cently, Wu and colleagues (9,10) showed that AGEs induce iNOS expression in RAW 264.7 cells through tyrosine kinase, phosphatidylinositol 3-kinase, p38MAPK, and PKC.

HO-1 is known as an oxidative-responsive gene and is induced by heme, heavy metals such as cadmium chloride, and depletion of intracellular-reduced glutathione, which generate reactive oxygen species and disturb the redox balance (15). The protective effects of HO-1 have been demonstrated as HO-1–mediated inhibition of atherosclerosis development in LDL receptor–deficient mice (37) and apoE-deficient mice (16,17). However, whether AGEs stimulate HO-1 expression in macrophages was unknown.

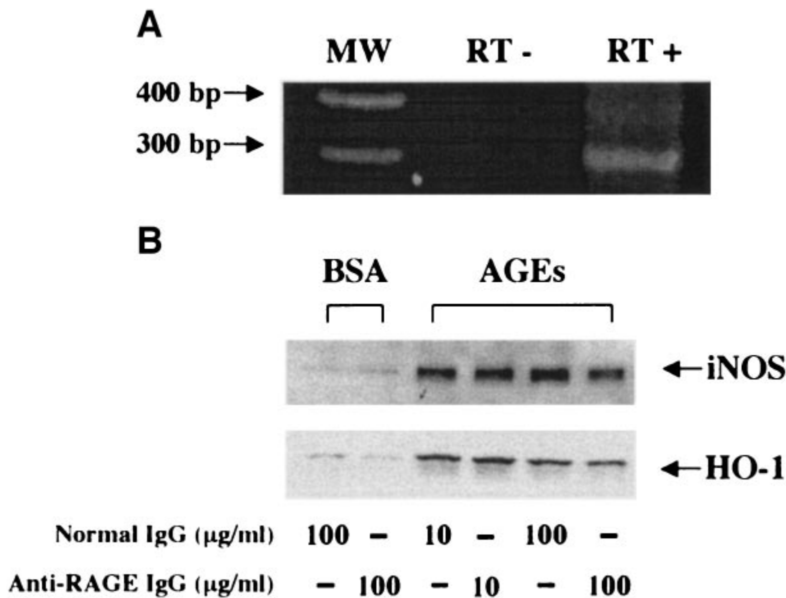

FIG. 6. AGE-induced iNOS and HO-1 protein expression in the presence of the anti-RAGE antibody. ***A*****: RT-PCRs were performed as described in RESEARCH DESIGN AND METHODS.** ***B*****: Cells were preincubated for 1 h with normal goat IgG or anti-RAGE antibody (10 and 100 μg/ml), followed by incubation with BSA or AGEs (33.2 μg/ml) for 12 h. Western blots were performed. MW, DNA size marker.**

FIG. 7. AGE-induced iNOS expression in the presence of the NO synthase inhibitor L-NAME. Cells were incubated with BSA, AGEs (33.2 μg/ml), or AGEs (33.2 μg/ml) plus L-NAME (1 mmol/l) for 0–36 h. Nitrite measurement (*A*) and Western blots (*B*) were performed. $*P < 0.05$ vs. AGEs.

In this study, we observed that AGEs induce iNOS and HO-1 expression in RAW 264.7 cells. In studying the mechanism of AGE-mediated iNOS and HO-1 induction, we show the involvement of p42/44 and p38 MAPK phosphorylation. The activation of p42/44 MAPK and PKC led to iNOS expression, activity, and NO production, whereas the activation of p38 MAPK caused induction of HO-1 expression. The expression of both proteins is regulated by NADPH oxidase, as acetovanillone inhibited AGE-induced iNOS and HO-1 expression. AGE-stimulated iNOS

FIG. 8. AGE-induced iNOS protein expression in the presence of the HO-1 inhibitor Tin-PP. Cells were preincubated for 3 h in the presence or absence of cadmium chloride (Cd; 25 μmol/l), followed by incubation with BSA or AGEs (33.2 μg/ml) for the indicated times. Nitrite measurement (*A*) and Western blots (*B*) were performed. $*P < 0.05$ vs. AGEs. *C*: Cells were preincubated for 9 h in the presence or absence of Tin-PP (Tin-PP; 20 or 50 μmol/l), followed by incubation with cadmium chloride (Cd; 25 μmol/l) for 3 h. Washed cells were incubated with BSA or AGEs (33.2 μg/ml) for 12 h. Western blots were performed. *D*: iNOS band intensities were quantified using NIH Image software. Data are presented as percentage of protein expression in the absence of Tin-PP. $*P < 0.05$ vs. without Tin-PP.

expression was downregulated by endogenous NO and HO-1 activity.

In the present study, we demonstrate that AGEs induce the phosphorylation of p42/44 and p38 MAPK. Our data are consistent with previous reports that AGEs phosphorylate p42/44 and p38 MAPK (38). Preincubation with inhibitors of p42/44, p38 MAPK, and PKC modulated AGE-stimulated nitrite accumulation, iNOS activity, and iNOS and HO-1 expression. p42/44 MAPK and PKC upregulated, while p38 MAPK downregulated, iNOS expression. In agreement with our findings, p38 MAPK was reported to downregulate iNOS expression in LPS plus IFN-γ–stimulated RAW 264.7γNO(−) cells (39) and IL-1β–induced mesangial cells (40). [RAW 264.7γNO(−) cells are a subclone of RAW 264.7 cells that do not express iNOS in response to IFN-γ alone.] However, other studies showed a stimulatory effect (41,42). Ajizian et al. (41) showed that p42/44 MAPK upregulates iNOS expression in LPS plus IFN-γ–stimulated mouse macrophages, and Bhat et al. (42) showed the same effect in LPS-induced glial cells. In addition, iNOS expression was upregulated by p38 MAPK independent of p42/44 MAPK in LPS-induced RAW 264.7 cells (32) and AGE-induced RAW 264.7 cells (9). As an explanation for these conflicting reports, we propose that differences in cell type, density, activator, or cell culture conditions may affect experimental results.

Recently, p38 MAPK activation has been associated with HO-1 expression. Ning et al. (43) showed that p38 MAPK is involved in the induction of HO-1 expression after transforming growth factor-β1 stimulation, and Buckley et al. (44) showed a role for p38 MAPK in HO-1 expression after exogenous NO addition. We also observed that AGEs induce HO-1 expression through the p38 MAPK pathway. This induction is further modulated by redox imbalance, since AGEs stimulate NADPH oxidase activity (27), and we show that the NADPH oxidase inhibitor (acetovanillone) inhibited AGE-induced HO-1 expression (Fig. 4). In addition, it has been reported that exogenous and endogenous NO stimulate induction of HO-1 expression in certain cell types (15). In the present study, endogenous NO did not induce HO-1 expression in AGE-stimulated RAW 264.7 cells. This is consistent with findings showing that exogenous NO could induce HO-1 expression but that endogenous NO produced in response to cytokines (IFN-γ and IL-1β) could not induce HO-1 expression in A172 human glioblastoma cells (45). It is possible that HO-1 induction by endogenous NO may not occur until after 36 h of AGE incubation. Nitrite accumulation was observed to peak after 36 h of AGE incubation. HO-1 expression may be induced through gene transcription and/or mRNA stabilization in response to NO after 36 h of AGE incubation.

It has been previously observed that iNOS-derived NO can downregulate both iNOS expression and activity after incubation by LPS or cytokines (13,46). NO may mediate this by inhibiting NF-κB through the induction and/or stabilization of IκBα (46) and/or directly interacting with the heme bound to iNOS (47). AGE-induced iNOS expression was negatively regulated by NO or its metabolites, with the strongest effect observed after 24 and 36 h of stimulation. We believe that after 12 h of AGE stimulation, NO concentrations are not yet high enough to influence iNOS expression.

We investigated whether HO-1 has a similar suppressive effect on AGE-stimulated iNOS expression. In previous reports, HO-1 expression induced by the incubation of heme (21) and 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (22) inhibits iNOS expression induced with a stimulator such as LPS. We observed a downregulation of AGE-stimulated iNOS expression by preinduction of HO-1 with cadmium chloride, indicating that HO-1 activity is required for downregulation of iNOS because Tin-PP prevented this effect. This observation may also involve other factors such as IL-10, which is regulated by CO (18), and other proteins induced by cadmium chloride.

We have yet to determine the mechanism by which AGEs activate NADPH oxidase but speculate that AGEs stimulate the translocation of the cytosolic components of NADPH oxidase to the plasma membrane, a necessary step in enzyme activation. This translocation requires the phosphorylation of $p67^{phox}$, $p47^{phox}$, and/or $p40^{phox}$. It has been reported that p42/44 (24), p38 MAPK (25), and PKC (26) phosphorylate cytosolic components of NADPH oxidase. In view of our findings that AGEs phosphorylate p42/44 and p38 MAPK and PKC, as part of the induction of iNOS expression, we propose that AGEs stimulate phosphorylation of the cytosolic components of NADPH oxidase through p42/44, p38 MAPK, and/or PKC, leading to enzyme activation. It is possible that NADPH oxidase activation could stimulate iNOS and HO-1 expression through ROS generation and redox imbalance. The inhibition of iNOS and HO-1 protein expression by acetovanillone was observed at relatively high concentrations (250 μmol/l to 2 mmol/l). These concentrations are similar to previous studies in which phorbol ester–induced endothelial leukocyte adhesion molecule-1 expression in endothelial cells (48) and tumor necrosis factor-α–induced monocyte colony-stimulating factor expression in mesangial cells (49) were examined.

We found that AGE-induced iNOS and HO-1 expression were downregulated by preincubation with anti-RAGE antibody. However, in both cases, expression was not completely neutralized with the anti-RAGE antibody. This suggests that there are other receptor pathways in addition to RAGE that are present in AGE-induced iNOS and HO-1 expression or, possibly, that a higher antibody titer is required for complete neutralization of endogenous RAGE.

In this study, AGEs caused the induction of iNOS and HO-1 expression through MAPKs and PKC. In addition, AGE-induced iNOS expression appears to be regulated by HO-1 and endogenous NO. Carbon monoxide and bilirubin derived from HO-1 activity appear to have antiatherosclerotic or anti-inflammatory effects. In our study, HO-1 activity suppressed iNOS expression, leading to a reduction in NO production. These results suggest that HO-1 activity may have a profound effect on the balance of NO and reactive oxygen species. We hypothesize that stimulation of HO-1 expression may represent a new strategy for the treatment of diabetic atherosclerosis. The identification of the mechanisms by which AGEs induce HO-1 and iNOS expression, as well as the interplay between these enzymes and their product, may be of relevance to the

development of a better understanding of diabetic atherosclerosis and nephropathy.

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