Original Article

Anti-Inflammatory Effects of the Advanced Glycation End Product Inhibitor LR-90 in Human Monocytes

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Ligation of advanced glycation end products (AGEs) with their receptor (RAGE) plays an important role in the development of various diabetes complications, including atherosclerosis. Monocyte activation, adhesion, and migration are key events in the pathogenesis of atherosclerosis. Previous studies showed that AGEs and S100b, a specific RAGE ligand, could augment monocyte inflammatory responses via RAGE. In this study, we examined whether LR-90, a compound belonging to a new class of AGE inhibitor, could inhibit inflammatory responses in human monocytes. Human THP-1 cells were pretreated with LR-90 and then stimulated with S100b. LR-90 significantly inhibited S100b-induced expression of RAGE and other proinflammatory genes including monocyte chemoattractant protein-1, interferon-γ–inducible protein-10, and cyclooxygenase-2 in a dose-dependent manner. These inhibitory effects may be exerted via inhibition of nuclear factor-κB (NF-κB) activation, as LR-90 suppressed both S100b– and tumor necrosis factor-α–induced IκB-α degradation as well as NF-κB promoter transcriptional activity. LR-90 also prevented oxidative stress in activated monocytes, as demonstrated by its inhibitory effects on S100b-induced expression of NADPH oxidase and intracellular superoxide production. In addition, LR-90 blocked S100b-induced monocyte adhesion to human umbilical vein endothelial cell. These new data show that, in addition to its AGE inhibitory effects, LR-90 has novel anti-inflammatory properties and might therefore have additional protective effects against diabetic vascular complications. *Diabetes* 56:647–655, 2007

Advanced glycation end products (AGEs), the products of nonenzymatic glycation and oxidation of proteins and lipids, accumulate in diverse biological settings, such as diabetes, inflammation, renal failure, and aging (1,2). AGEs have been proposed to play a crucial role in the pathogenesis of diabetic vascular complications (3,4). Patients with diabetes exhibit an increased propensity to develop atherosclerosis that can lead to severe complications such as acute stroke, myocardial infarction, and peripheral vascular disease (4,5).

The cellular effects of AGEs are mediated by specific receptors, one of which is the receptor for AGE (RAGE). The presence of RAGE has been demonstrated in all cells relevant to the atherosclerotic process including monocytes, macrophages, endothelial cells, and smooth muscle cells (6). These vascular cells do not express significant amounts of RAGE under physiological conditions but can be induced to express RAGE in situations where either ligands accumulate and/or various transcription factors regulating RAGE are activated (7). In the diabetic vasculature, cells expressing high levels of RAGE are often proximal to or colocalized in areas in which AGEs are abundant (8,9). Aside from AGEs, tumor necrosis factor-α (TNF-α), S100/calgranulins, β-amyloid, amphoterin, and oxidative stress can upregulate RAGE expression, thus rendering these cells more susceptible to the effects of AGEs and chronic inflammation (7,10,11). The role of RAGE in the pathogenesis of atherosclerosis has been reviewed recently (11–14). Cell and animal studies suggest that limiting RAGE expression in vascular cells could modulate expression of various proinflammatory mediators and prevent atherosclerosis development (15–18). Thus, targeting RAGE is a promising intervention for the treatment of human diabetic vascular disease and inflammatory disorders (19).

Previously, our group reported new classes of compounds as inhibitors of AGE formation and protein crosslinking (20). More recently, several LR compounds, specifically LR-90, methylene bis [4,4-(2 chlorophenylureido phenoxyisobutyric acid)], were found to be effective in preventing the development of diabetic nephropathy in streptozotocin-induced diabetic animals (21). Additionally, we have found that LR-90 exhibits vascular protective properties in the aorta of diabetic animals (S.R., unpublished observations). In the present study, we hypothesized that LR-90 can inhibit inflammatory responses in monocytes treated with S100b, a specific RAGE ligand, or TNF-α. We therefore evaluated the effects of LR-90 on the expression of various inflammatory mediators associated with monocyte activation and adhesion. Our results demonstrate for the first time that LR-90 possess anti-inflammatory properties, as it effectively prevented S100b-induced expression of RAGE and other inflammatory genes in THP-1 cells and also inhibited monocyte binding to endothelial cells in a dose-dependent manner. Furthermore, we also show that the anti-inflammatory effects of LR-90 might

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AGE, advanced glycation end product; COX-2, cyclooxygenase-2; DHE, dihydroethidine; DPI, diphenyleneiodonium; ELISA, enzyme-linked immunosorbent assay; HUVEC, human umbilical vein endothelial cell; IP-10, interferon-γ–inducible protein-10; MCP-1, monocyte chemoattractant protein-1; NF-κB, nuclear factor-κB; NOX2, NADPH oxidase; RAGE, receptor for AGE; ROS, reactive oxygen species; TNF-α, tumor necrosis factor-α.

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TABLE 1
Primer sequences and PCR products

Target gene	PCR primer sequence (5′ to 3′)	PCR product (bp)
RAGE	F: AGAGGAGAGGAAGGCCCCAGA R: GGCAAGGTGGGGTTATACAGG	220
MCP-1	F: GCCTTAAGTAATGTTAATTCTTAT R: GGTGTAATAGTTACAAAATATTCA	239
IP-10	F: TGAAAAAGAAGGGTGAGAAGAG R: GGAAGATGGGAAAGGTGAGG	413
COX-2	F: ATCTACCCTCCTCAAGTCCC R: TACCAGAAGGGCAGGATACAG	708
NOX-2	F: GGAGTTTCAAGATGCGTGGAAACTA R: GCCAGACTCAGAGTTGGAGATGCT	549

F, forward; R, reverse.

be mediated by its ability to prevent oxidative stress and modulate NF-κB activation in THP-1 cells.

RESEARCH DESIGN AND METHODS

LR-90, LR-51, and LR-74 were synthesized by Dr. I. Lalezari (Chemiphar, Scarsdale, NY). Cell culture reagents were from Invitrogen (Carlsbad, CA) and Cascade Biologics (Portland, OR). S100b was obtained from Calbiochem (La Jolla, CA). TNF-α and Quantikine monocyte chemoattractant protein-1 (MCP-1) enzyme-linked immunosorbent assay (ELISA) kit were from R&D Systems (Minneapolis, MN). RNA-Stat60, Quantum RNA 18S primers, and RT-PCR kits were purchased from Tel-Test (Friendswood, TX), Ambion (Austin, TX), and Applied Biosystems (Foster City, CA), respectively. BAY 11-7082 [(E)-3-(4-Methylphenylsufonyl)-2-propenenitrile] was purchased from Axxora (San Diego, CA). Anti–IkB-α, anti-p65, and anti-histone H2B antibodies were obtained from Cell Signaling (Beverly, MA). MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay kit was purchased from Chemicon (Temecula, CA). All other reagents were purchased from Sigma (St. Louis, MO).

Cell culture and treatment. Human THP-1 monocytic cells were obtained from the American *Type Culture* Collection (Manassas, VA) and maintained in RPMI-1640 medium supplemented with 10% heat-inactivated FCS, β-mercaptoethanol (50 μmol/l), HEPES (10 mmol/l), glutamine (2 mmol/l), streptomycin (50 μg/ml), penicillin (50 units/ml), and glucose (5.5 mmol/l) in a 5% CO_2 incubator at 37°C. THP-1 monocytes (5×10^5 cells/ml) were pretreated with various concentrations of LR-90 (0, 25, 50, 100, and 200 μmol/l; prepared in DMSO) for 1 h. Control cells received vehicle only (<0.5% DMSO). Thereafter, S100b (50 μg/ml) was added and cells reincubated for 4 h. The cells were harvested by brief centrifugation, and RNAStat-60 was added to the pellet before storing at 70°C for subsequent RNA extraction and analyses. For the NF-κB inhibition experiment, THP-1 monocytes were cultured in serum-depleted RPMI medium for 24 h. Cells were pretreated with LR-90 or BAY 11–7082 (NF-κB inhibitor) for 1 h and then stimulated with or without S100b (50 μg/ml) or TNF-α (20 ng/ml) for 60 and 15 min, respectively. After incubation, nuclear and cytosolic extracts were isolated as described below. Human umbilical vein endothelial cells (HUVECs) were purchased from Cascade Biologics (Portland, OR) and cultured according to the manufacturer's specifications.

RNA preparation and relative RT-PCR. Total RNA was isolated by the RNAStat-60 method, and 1 μg was used for the RT reaction using a Gene AmpRNA PCR kit. Total cDNA corresponding to 0.05 μg RNA was then used in PCRs containing gene-specific primers (Table 1) paired with Quantum RNA 18S internal standards. Multiplex PCRs were performed in a GeneAmp 9700 (Applied Biosystems). PCR products were fractionated on 2.0% agarose gel electrophoresis and photographed using an AlphaImager 2000 Documentation and Analysis system (Alpha Innotech, San Leandro, CA). The densities of amplified products corresponding to specific genes and 18S RNA were quantified with Quantity One software (Bio-Rad, Hercules, CA). Results are expressed as fold stimulation over control after normalizing with the internal standard (18S RNA).

Protein extraction and Western blotting. Cytosolic and nuclear extracts were prepared and subjected to electrophoresis on 12.5% SDS-polyacrylamide gels, transferred to nitrocellulose membrane, and probed with anti–IκB-α and anti-p65 antibodies, respectively. Immunoreactive proteins were detected by enhanced chemiluminescence (SuperSignal West Pico Chemiluminescent Substrate; Pierce, Chicago, IL) using horseradish peroxidase–labeled anti-rabbit antiserum. Blots were exposed to film for 10 s to 2 min and then developed. To ensure equal loading of proteins, membranes were stripped and restained with antibodies against β-actin and histone H2B.

Measurement of MCP-1 protein levels by ELISA. THP-1 cells maintained in serum-free RPMI medium containing 0.2% BSA were pretreated with LR-90 for 1 h before 18 h of stimulation with S100b (50 μg/ml). ELISA for MCP-1 protein was performed on cell-free supernatants using the Quantikine ELISA kit according to the manufacturer's protocol.

Transient transfections and luciferase assays. THP-1 cells (1.0×10^6 per ml) were transfected with pNF-κB-Luc plasmid (Stratagene, LA Jolla, CA) using the Amaxa transfection kit (Amaxa, Gaithersburg, MD) according to manufacturer's protocols. Transfection efficiency was monitored by examining green fluorescent protein expression (~60% transfection efficiency). The transfected cells were pooled together and allowed to recover overnight. An equal number of cells were plated on 12-well culture plates and pretreated with various concentrations of the test compounds for 1 h and then stimulated with S100b for 4 h. Cells were harvested, washed with ice cold PBS, and lysed with 100 ml reporter lysis buffer, and 25 μl of each lysate was analyzed for luciferase activity using the Promega Luciferase Assay System (Promega, Madison, WI). Relative luciferase activities were normalized to the protein concentration of each sample.

Determination of intracellular superoxide production. S100b-induced superoxide generation was detected using the fluorescence marker dihydroethidine (DHE) staining. Cells are permeable to DHE, which in the presence of superoxide is oxidized to fluorescent ethidium bromide. Ethidium bromide is trapped by intercalation with DNA, and the number of fluorescent nuclei indicates the relative level of superoxide production. THP-1 monocytes (5×10^5 cells/ml) were seeded on a 12-well plate in serum-free RPMI medium containing 0.2% BSA for 24 h and then pretreated with LR-90 for 1 h. Then, S100b was added to each well for 30 min and stained with DHE (10 μmol/l) for 15 min at 37°C. After incubation, cells were collected, washed, and resuspended in PBS, and then transferred to a 24-well plate. Fluorescence was detected using a confocal laser-scanning microscope (wavelength 510/595 nm), and the number of fluorescence cells was counted and analyzed using Image Pro software (Media Cybernetics, Silver Spring, MD). Results were presented as fold of superoxide production over control.

Monocyte adhesion assay. THP-1 monocytes (5×10^5 cells/ml) cultured in 10% FCS/RPMI medium were pretreated with LR-90 for 1 h and then stimulated with S100b for 24 h. After the incubation period, cells were washed three times with serum-free RPMI medium and resuspended in the same medium before adding calcein-AM for 1 h at 37°C. After incubation, cells were washed, and 1×10^5 cells were added to HUVECs (80% confluence) seeded previously for 24–48 h in a 24-well plate. Monocytes were allowed to bind to HUVEC for 2 h before carefully washing all of the nonspecifically bound cells in each well. Bound monocytes were detected using fluorescent microscopy and counted per microscope image field (three random fields per treatment) using Quantity One software. Results were expressed as the number of bound monocytes per field.

Cell viability test. THP-1 cells were seeded onto 12-well plates at a concentration of 5×10^5 cells/ml in RPMI medium containing 10% FCS and then incubated with various concentrations of LR-90, vehicle (DMSO), or control medium alone for 24 h at 37°C, 5% CO_2. After incubation, overall cell counts were measured using a cell counter (Coulter Z1; Beckman Coulter, Fullerton, CA), and the number of viable cells was determined by hemocytometer counts of Trypan Blue–impermeable cells. In another test, cell viability was determined using the colorimetric MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay kit following the manufacturer's instructions.

Data analyses. Statistical analyses were performed using Prism software (GraphPad, San Diego, CA). Unless otherwise indicated, data are presented as means ± SE. Group comparisons were analyzed using one-way ANOVA and subsequent post hoc analysis by unpaired Student's *t* test. A P value <0.05 was considered statistically significant.

RESULTS

LR-90 inhibits the expression of RAGE and various proinflammatory genes in S100b-stimulated monocytes. THP-1 cells incubated with S100b for 4 h showed an approximate fourfold increase in mRNA expression of RAGE (Fig. 1*A*). Similarly, we observed significant upregulation of the chemokines MCP-1 (Fig. 1*B*) and interferon-γ–inducible protein-10 (IP-10) (Fig. 1*C*), as well as the inflammatory enzyme cycloxygenase-2 (COX-2) (Fig. 1*D*). When THP-1 monocytes were pretreated with LR-90 for 1 h

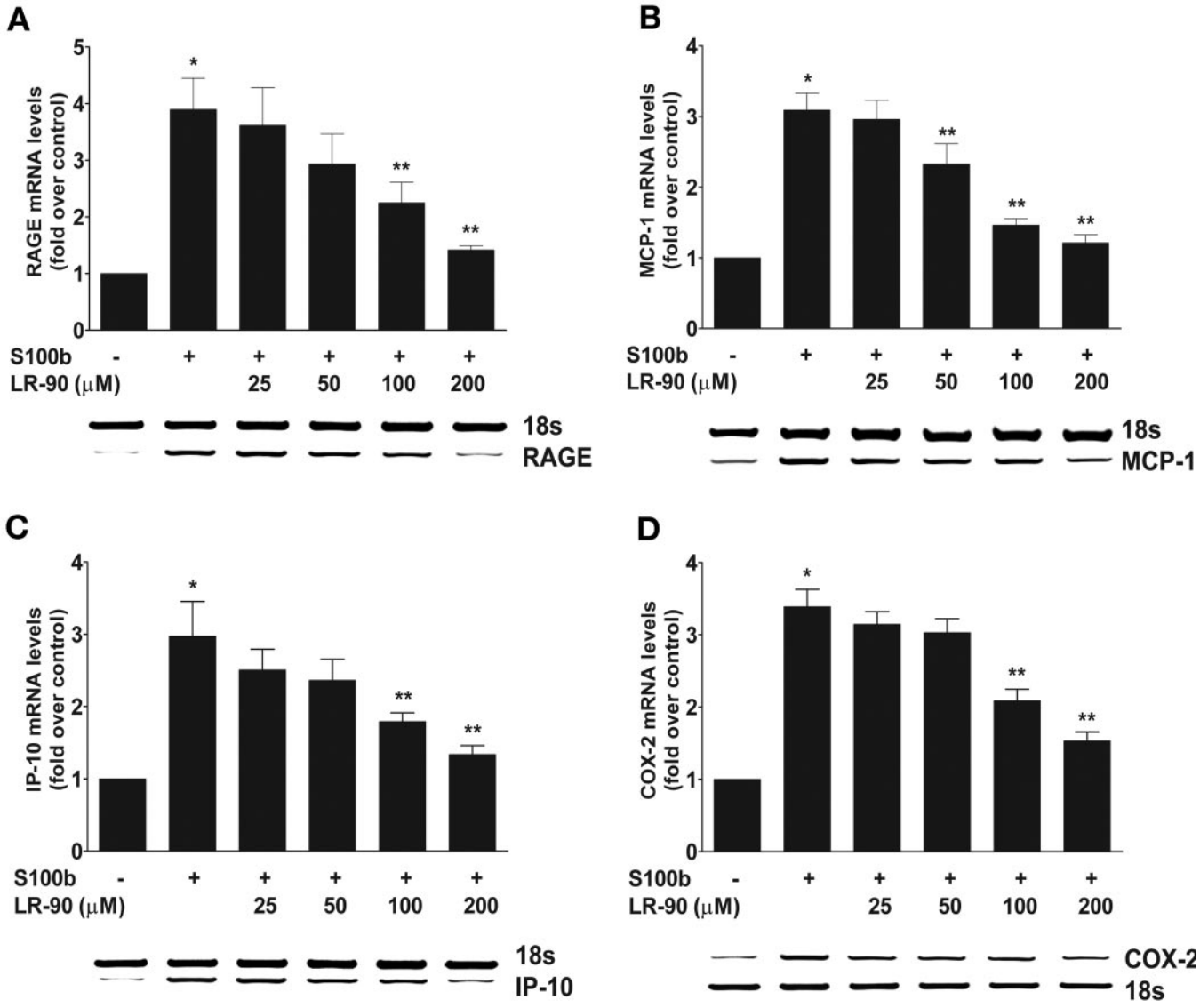

FIG. 1. LR-90 dose-dependently inhibits mRNA expression of RAGE, MCP-1, COX-2, and IP-10 in human monocytes stimulated with S100b. THP-1 cells were pretreated with or without LR-90 for 1 h before they were stimulated with S100b for 4 h. After treatment, relative RT-PCRs were performed with total RNA isolated from these cells using gene-specific primers. PCR products were analyzed on 2% agarose gel electrophoresis, photographed, and quantified using an image analysis program. The bar graph shows fold-stimulation over control and represents the mean ± SE of three independent experiments. Image below each graph shows a representative RT-PCR experiment. *$P < 0.05$ vs. control; **$P < 0.05$ vs. S100b-stimulated cells.

before S100b stimulation, there was profound suppression of the expression of all of these genes in a dose-dependent manner. LR-90 concentrations of 100 and 200 μmol/l significantly reduced mRNA expression of all of the genes analyzed ($P < 0.05$). These data suggest that LR-90 can inhibit the proinflammatory effects of S100b by blocking cellular mediators known to be involved in atherosclerosis.

LR-90 inhibits MCP-1 protein expression. MCP-1 plays an important role in monocyte chemotaxis and vascular disease, including atherosclerosis. To examine whether the decrease in MCP-1 gene expression by LR-90 treatment observed in the RT-PCR experiment above could result to reduced MCP-1 protein expression, we treated THP-1 cells with or without LR-90 and then stimulated the cells with S100b for 18 h. MCP-1 levels in culture supernatants were quantified by ELISA. S100b treatment led to almost a 10-fold increase in MCP-1 protein expression after 18 h (Fig. 2). This increase was inhibited by pretreatment with LR-90 in a similar dose-dependent manner, with 100 and 200 μmol/l LR-90 significantly decreasing the protein levels by >50 and 70%, respectively. These data indicate the LR-90 can reduce MCP-1 mRNA as well as protein expression.

LR-90 suppresses S100b-induced NOX2 activation and oxidant stress in monocytes. Evidence shows that the ligation of RAGE can induce oxidant stress via stimulation of redox-sensitive NADPH oxidase leading to superoxide anion generation (22). We therefore examined whether LR-90 can also suppress the function of this enzyme and the resulting intracellular superoxide production by DHE staining. NADPH oxidase is a multi-subunit enzyme, and the $gp91^{phox}$ subunit (NOX2) functions as the catalytic core for the electron transfer to form superoxide anion (23,24). THP-1 cells stimulated with S100b showed significant threefold increase in NOX2 gene expression compared with untreated cells as detected by RT-PCR (Fig. 3*A*). Pretreatment of cells with LR-90 dose-dependently inhibited NOX2 expression. Preincubation with diphenyleneiodonium (DPI), an inhibitor of NADPH oxi-

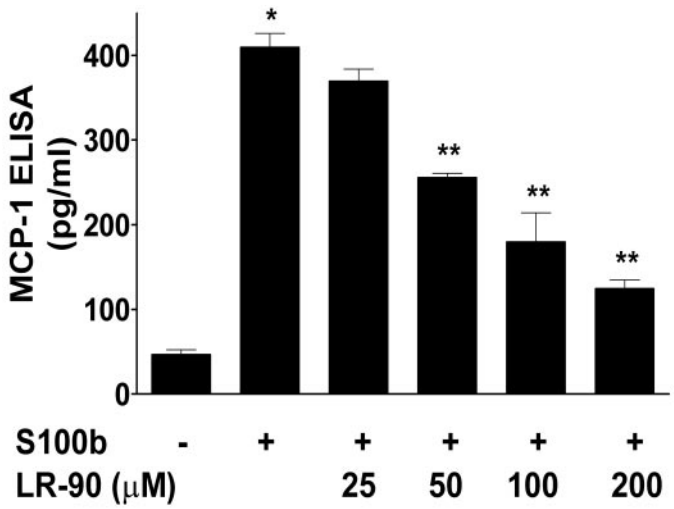

FIG. 2. Effects of LR-90 on MCP-1 protein expression. THP-1 cells cultured in serum-free media for 24 h were treated with or without LR-90 for 1 h before they were stimulated with S100b for 18 h. After treatment, MCP-1 ELISA was performed on cell-free supernatants. Values shown are mean ± SE of multiple independent experiments. *$P < 0.05$ vs. control; **$P < 0.05$ vs. S100b-stimulated cells.

dase, almost completely suppressed induction of NOX2 by S100b. LR-74, a similar AGE inhibitor with potent metal chelating activity (20), also inhibited NOX2 expression. Interestingly, Tiron (1,2-dihydroxy-3, 5-benzenedisulfonic acid), a metal chelator and superoxide scavenger, showed almost no inhibitory effects. There was also no inhibition seen with LR-51, a related LR compound with weak AGE inhibitor properties and metal chelating characteristics.

Consistent with the above observations, S100b stimulation also significantly induced the production of NADPH-dependent intracellular superoxide production (>10-fold) relative to untreated cells (Fig. 3*B* and *C*). Pretreatment with LR-90 significantly reduced superoxide levels in a similar dose-dependent manner. Preincubation with either DPI or Tiron almost completely abolished superoxide production. LR-74 also prevented superoxide generation but not LR-51. Similar results were observed when THP-1 cells were treated with TNF-α (data not shown). Taken together, these results indicate that LR-90 can inhibit oxidative stress in monocytes induced by inflammatory stimuli by blocking the expression of NOX2 and preventing superoxide production.

LR-90 inhibits S100b– and TNF-α–induced NF-κB activation. NF-κB is a key oxidant-sensitive transcription factor that can mediate the expression of several proinflammatory genes including MCP-1, RAGE, IP-10, and COX-2. We therefore examined whether LR-90's actions may be mediated via inhibition of NF-κB activity. Activation of NF-κB involves phosphorylation, ubiquitination, and degradation of its cytoplasmic inhibitor IκB-α subunit. This phosphorylation and degradation of IκB-α allows NF-κB to translocate to the nucleus where it can activate gene transcription (25). Thus, degradation of IκB-α is a key marker for the activation of NF-κB. To determine whether LR-90's anti-inflammatory activity is associated with blocking of NF-κB activation, THP-1 monocytes were treated with either S100b (60 min) or TNF-α (15 min), and cytoplasmic and nuclear fractions were assayed by Western blotting with specific anti–IκB-α and anti-p65 antibodies. Treatment of THP-1 cells with S100b (Fig. 4*A*, *upper panel*) or TNF-α (Fig. 4*A*, *middle panel*) led to a rapid loss of IκB-α from the cytoplasm and increased the levels of NF-κB transcriptionally active subunit p65 in the nuclear fractions (Fig. 4*A*, *lower panel*). Addition of BAY 11–7082, a known NF-κB inhibitor (26), stabilized IκB-α degradation induced by both activators (Fig. 4*A*, *lane 7*). Similarly, pretreatment of LR-90 dose-dependently prevented IκB-α degradation and translocation of NF-κB p65 to the nucleus (Fig. 4*A*, *lanes 3–6*). Lanes 8 and 9 of Fig. 4 show that the two inhibitors have no effects on their own. These data suggest that LR-90's anti-inflammatory effects on activated monocytes could be due to its negative effects on NF-κB activation.

To further confirm this inhibition of NF-κB activation, we investigated whether LR-90 could inhibit NF-κB promoter activity at the transcriptional level. THP-1 cells were transiently transfected with a plasmid containing a heterologous promoter driven by NF-κB elements upstream of the luciferase gene. As shown in Fig. 4*B* (*upper panel*), S100b treatment significantly increased the NF-κB promoter driven luciferase activity several-fold compared with control cells. BAY 11–7082 markedly inhibited this increased activity, as anticipated. Treatment with LR-90 dose-dependently reduced S100b-induced NF-κB transcriptional activity. Similar effects of LR-90 were observed when TNF-α was used to stimulate THP-1 monocytes (Fig. 4*B*, *lower panel*).

LR-90 prevents S100b-induced binding of THP-1 monocytes to HUVECs. To determine the functional significance of the inhibitory effects of LR-90 on various proinflammatory mediators with respect to monocyte activation, we next investigated whether LR-90 can prevent monocyte adhesion to endothelial cells. Monocyte binding to endothelial cells plays a primary role in monocyte recruitment to the vessel wall, a key initial event in the pathogenesis of atherosclerosis (27). Results show that S100b-treated THP-1 cells displayed a >10-fold increase in adherence to HUVECs relative to the untreated control cells (Fig. 5*A* and *B*). Pretreatment with LR-90 for 1 h before S100b stimulation significantly prevented monocyte adherence to endothelial cells in dose-dependent fashion with 100 and 200 μmol/l concentrations decreasing monocyte binding by >50% ($P < 0.05$).

Cell viability of LR-90–treated monocytes. To verify that the inhibitory effects of LR-90 on monocyte activation and adhesion is not secondary to cell toxicity, we treated THP-1 cells with similar concentrations of LR-90 used in all experiments and determined cell viability after 24 h of treatment. Cell counts for all concentrations tested remained statistically similar to the untreated cells after the incubation period (Fig. 6*A*). Furthermore, Trypan Blue staining (Fig. 6*B*) and MTT assays (Fig. 6*C*) both showed that the LR-90–stimulated cells remained viable after the 24 h.

DISCUSSION

There is a growing body of evidence, ranging from in vitro to in vivo experiments, and several pathologic analyses and epidemiological studies showing that atherosclerosis is a fundamentally inflammatory disease characterized by increased levels of adhesion molecules, cytokines, growth factors, and other related molecules (28,29). Monocytes are often described as the orchestrators of the inflammatory response and clearly play key roles in inflammatory process. Recruitment of monocytes and lymphocytes from the peripheral blood to the intima of the vessel wall is a

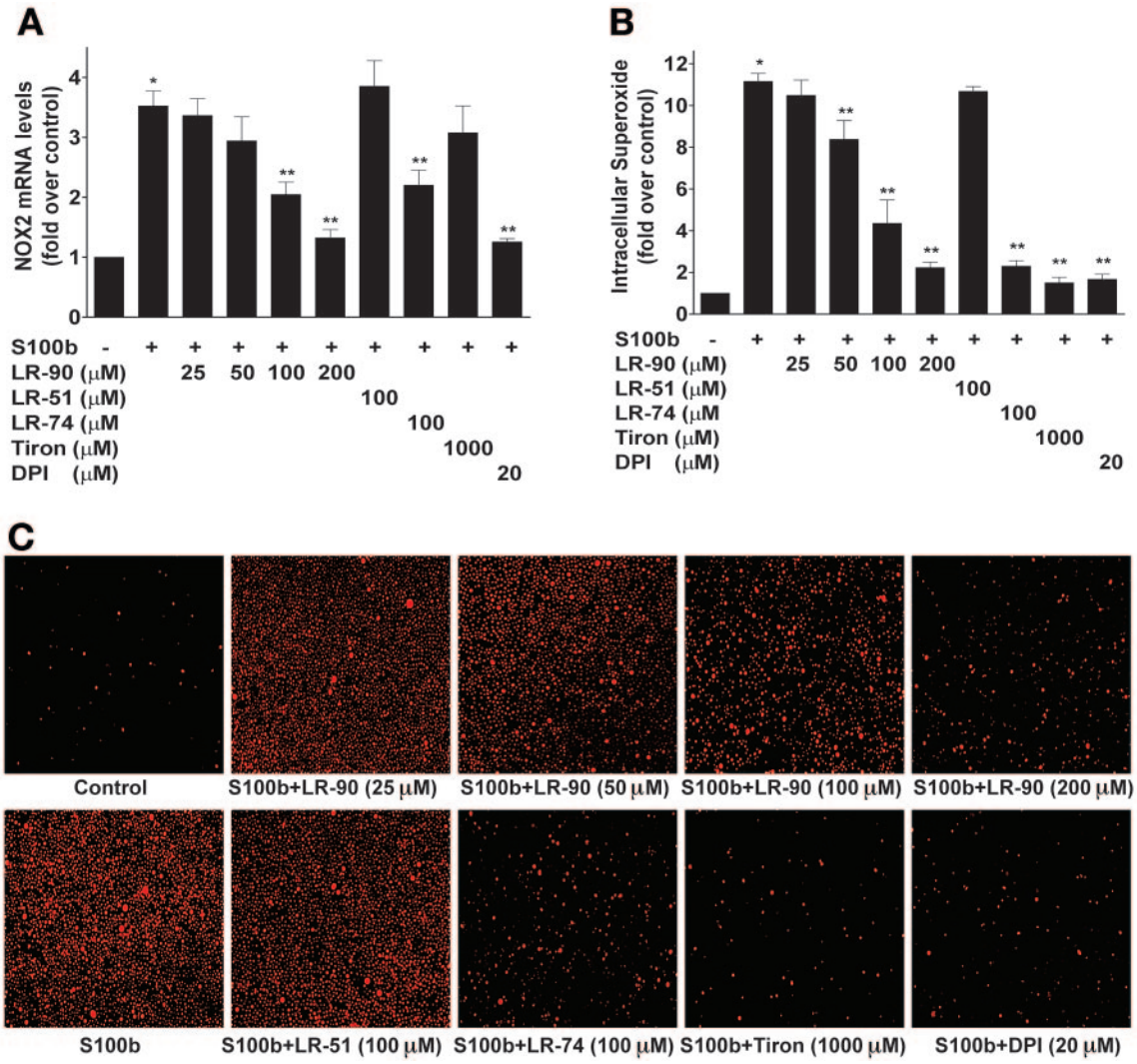

FIG. 3. LR-90 inhibits NOX2 expression and oxidative stress in THP-1 monocytes treated with S100b. *A*: Effects of LR-90 on NOX2 expression. THP-1 cells were pretreated with or without LR-90 for 1 h before they were stimulated with S100b for 4 h. After treatment, relative RT-PCR was performed with total RNA isolated from cells using specific primers for the gp91^{phox} (NOX2) catalytic subunit of NADPH oxidase. PCR products were analyzed on 2% agarose gel electrophoresis, photographed, and quantified using an image analysis program. The bar graph shows fold-stimulation over control and represents the mean ± SE of three independent experiments. *P < 0.05 vs. control; **P < 0.05 vs. S100b-stimulated cells. *B* and *C*: Effects of LR-90 on intracellular superoxide production. THP-1 cells cultured in serum-free medium for 24 h were pretreated with or without LR-90 for 1 h. S100b was then added to the culture medium for 30 min and the cells stained with DHE. The red fluorescence due to superoxide production was detected using a confocal fluorescence microscope and analyzed using an image analysis program (*B*). Representative photomicrographs from three independent experiments are shown in *C*.

central event in atherosclerosis (27). Animal studies have shown that the majority of the cells in the lipid core of atherosclerotic plaques are derived from monocytes (30). Earlier observations have shown that binding of soluble AGEs to RAGE on the surface of monocytes induces chemotaxis (31). Recently, we have demonstrated that high-glucose treatment, or ligation of RAGE by AGEs or S100b protein in THP-1 monocytes or human peripheral blood monocytes, leads to increased expression of a number of inflammatory cytokines, growth factors, and adhesion molecules and their receptors and inflammatory enzymes (32–34). In the present study, we demonstrated for the first time that LR-90 has potent anti-inflammatory effects on monocytes activated by S100b or TNF-α. Our results show that LR-90 dose-dependently blocked S100b-induced expression of RAGE and other proinflammatory molecules including MCP-1, IP-10, and COX-2 enzyme. Moreover, these anti-inflammatory effects were dose dependent, correlating with the observed repression of NF-κB activation. These findings are consistent with several studies in which inhibition of RAGE expression leads to attenuation of expression of several proinflammatory molecules associated with atherogenesis (17,18).

Ligation of RAGE leads to enhanced intracellular oxidative stress (22,35). One of the most immediate responses of monocytes to various stimuli including cytokines and RAGE activation is the production of superoxide anion, a potent free radical (36,37). Superoxide anion is a reactive oxygen species (ROS) intermediate, serving as starting material for the generation of a variety of other ROS species, as well as influencing ROS production by other enzymes, that can alter cell function including adhesion, proliferation, and motility (24). Superoxide can be generated by NADPH oxidase, which is composed of several subunits including gp91^{phox} (NOX2) (23,24). Ligation of RAGE with S100b induces monocytes to produce intracellular superoxide, as detected by DHE, a specific fluorescence probe. We used DPI (an NADPH oxidase inhibitor)

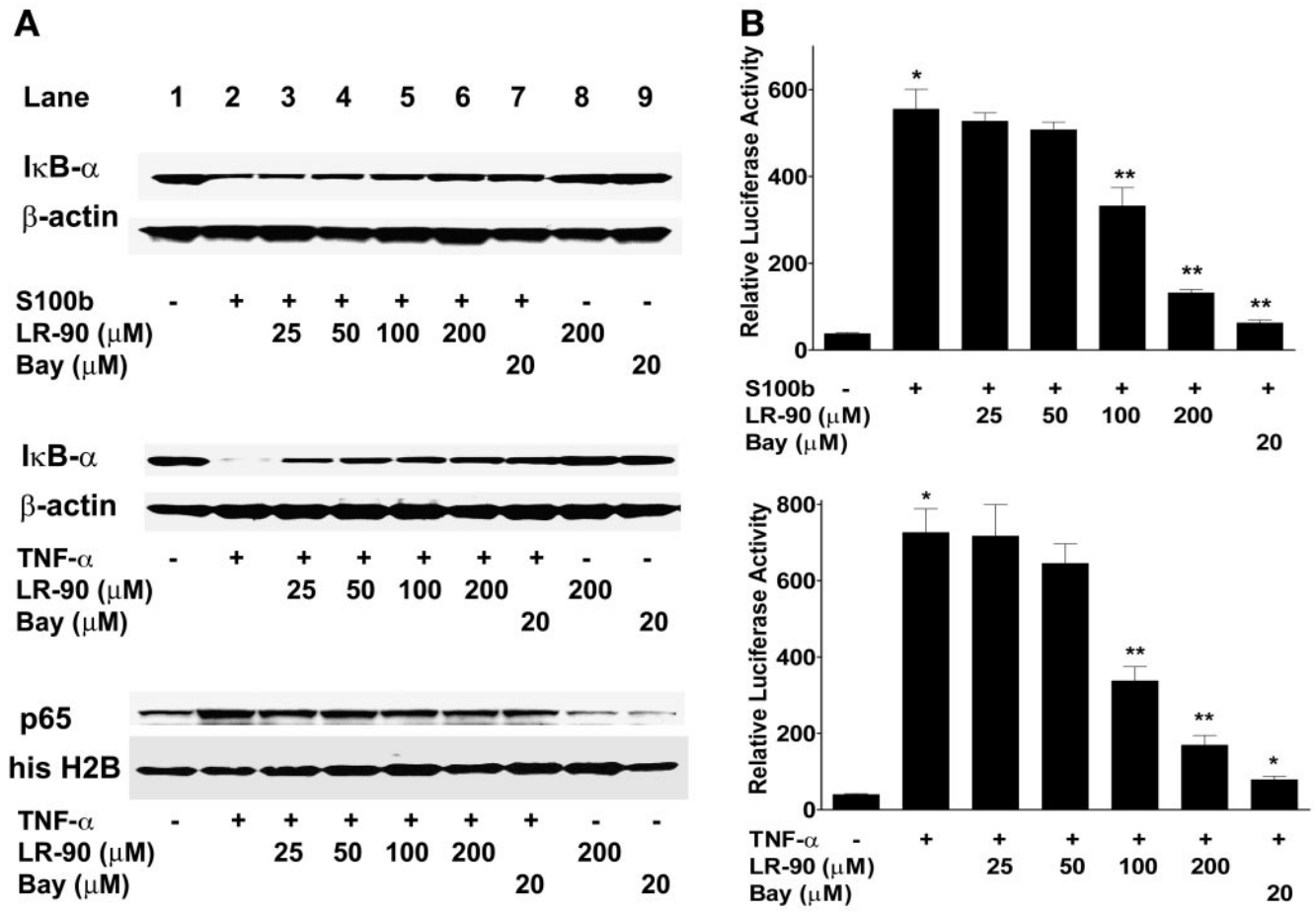

FIG. 4. Effects of LR-90 on NF-κB activation. *A*: LR-90 inhibits IκB-α degradation. THP-1 cells were pretreated with or without LR-90 for 1 h and then stimulated with TNF-α (20 ng/ml) or S100b (50 μg/ml) for 15 and 60 min, respectively. Cells were harvested, and cytoplasmic and nuclear proteins were extracted. Cytosolic and nuclear proteins (25 μg) were separated by SDS-PAGE, transferred to nitrocellulose membranes, and probed with anti–IκB-α and anti-p65 antibodies, respectively. Equal loading of protein was confirmed by stripping the membrane and staining with anti–β-actin or anti-histone H2B antibodies. Blots shown are from a typical Western blot experiment. *B*: Effects of LR-90 on S100b-induced NF-κB promoter activities in THP-1 cells. Cells were transfected with 0.5 μg pNF-κB-Luc reporter plasmid. After transfection, cells were subcultured in 12-well plates, pretreated with or without LR-90 for 1 h, and then stimulated with S100b (*upper panel*) or TNF-α (*lower panel*) for 4 h. Cells were harvested and the levels of luciferase activities determined as described in the RESEARCH DESIGN AND METHODS. Data represent results of three independent transfection experiments. *$P < 0.05$ vs. control; **$P < 0.05$ vs. S100b-stimulated cells.

and Tiron (a metal chelator and cell membrane permeable scavenger of superoxide) as positive controls. Our data clearly showed that all of the metal chelators and antioxidants, including LR-90, effectively suppressed intracellular superoxide production in S100b– and TNF-α–treated cells. Furthermore, this reduction in intracellular superoxide production by LR-90 was associated with blocking NADPH oxidase activity, since the expression of NOX2 was decreased in parallel in a dose-dependent manner. NOX2 expression in monocytes is 500-fold higher than in any other nonphagocytic cells, and its expression is directly related to the severity of atherosclerosis (38). Thus, our results clearly show that LR-90 prevents oxidative stress in activated monocytes. Interestingly, Tiron showed no inhibitory effect on NOX2 mRNA expression. This is consistent with published reports indicating that Tiron and other antioxidants directly scavenge and inactivate ROS rather than interfering with the expression of the oxidant enzymes (39). Although LR-90 has only moderate metal chelating activity (20), these results suggest that metal chelation is not solely responsible for the anti-inflammatory effects of LR-90 observed in our studies. A more recent study suggests that NF-κB itself is a key factor in controlling NOX2 expression and activation in monocytes, which can lead to further superoxide production by NADPH oxidase (40).

Activation of NF-κB leads to transcriptional activation of many genes involved in the inflammatory processes in atherosclerosis (41). In resting cells, NF-κB resides in the cytoplasm in an inactive form bound to inhibitor molecules, the IκB proteins. Regulation of this redox-sensitive transcription factor is controlled primarily through receptor-induced activation of the IκB kinase complex (IKK), leading to phosphorylation, proteoasome-dependent ubiquitination, and degradation of IκB proteins (25). The degradation of IκB results in the release and translocation of NF-κB to the nucleus, where it can bind and stimulate transcription of target genes such as cytokines, chemokines, growth factors, RAGE, and IkB-α (7,41). We have found previously that the enhanced expression of MCP-1 and COX-2 in high glucose and S100b-stimulated monocytes is mediated via NF-κB (32,33). Thus, inhibition of the NF-κB by LR-90 would predictably reduce NF-κB–mediated activation of these proinflammatory genes as observed in our experiments. Our results showed that LR-90 dose-dependently inhibited S100b– and TNF-α–induced degradation of IκB-α as well as p65 nuclear accumulation. Furthermore, LR-90 also inhibited NF-κB promoter tran-

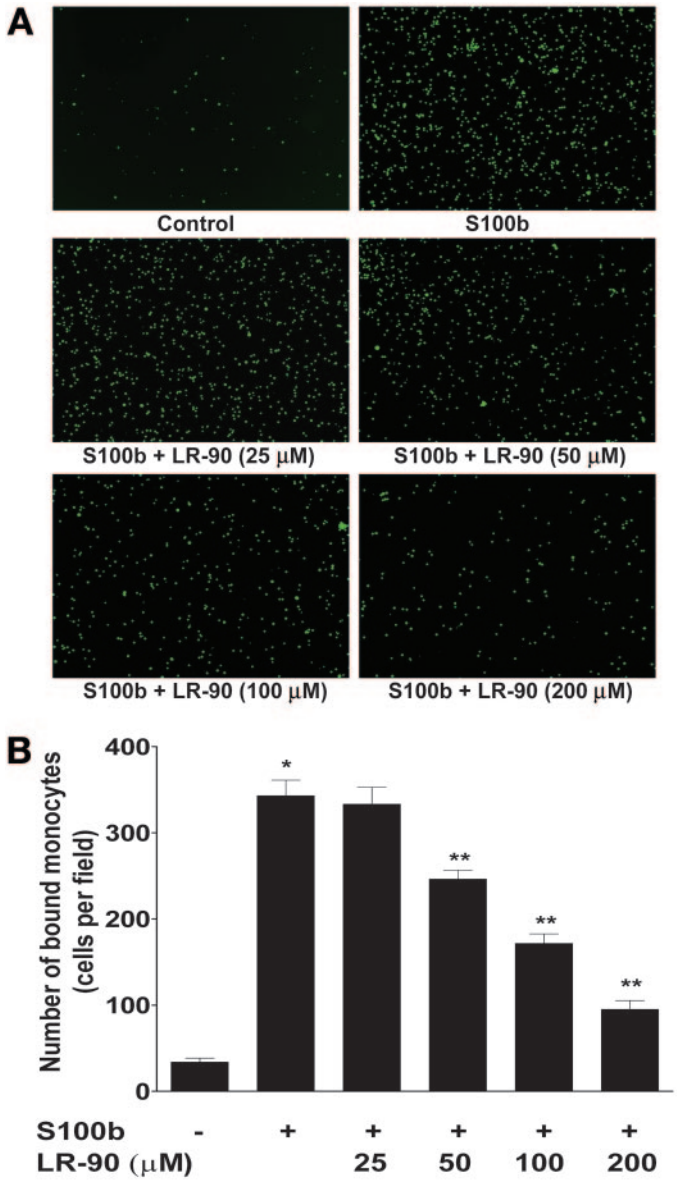

FIG. 5. LR-90 prevents monocyte adhesion to HUVECs. THP-1 cells were treated with or without LR-90 for 1 h and then stimulated with S100b for 24 h. After the incubation period, cells were labeled with calcein-AM and allowed to adhere to HUVECs for 2 h. Bound cells were detected using fluorescence microscope. *A*: Representative photomicrographs of the adhesion experiments. *B*: Mean ± SE counts of bound monocytes per image field for the four independent experiments. **P* < 0.05 vs. control; ***P* < 0.05 vs. S100b-stimulated cells.

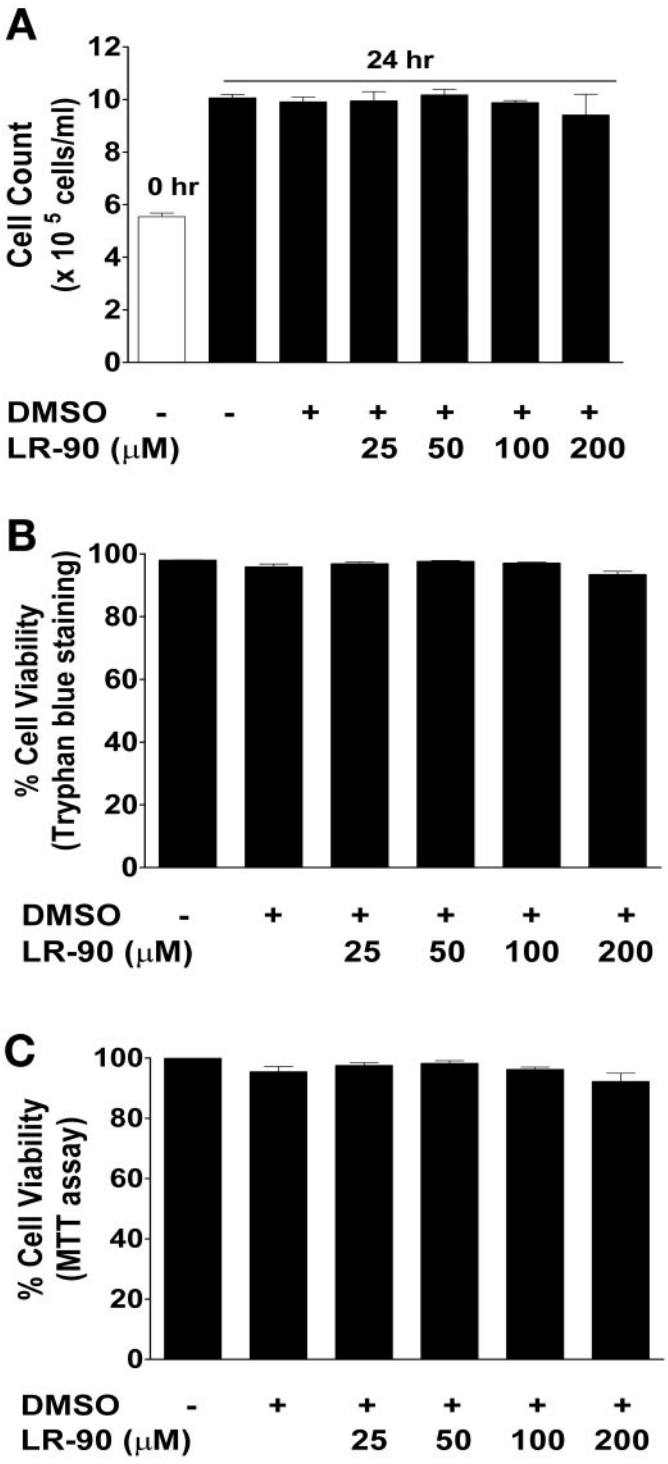

FIG. 6. Cell viability of monocytes treated with LR-90. THP-1 cells maintained in 10% FCS RPMI medium were treated with various concentrations of LR-90 and incubated at 37°C, 5% CO_2, for 24 h. After incubation, cells were counted using a cell counter (*A*), and the number of viable cells was determined by hemocytometer counts of Trypan Blue–impermeable cells (*B*) or by MTT staining assay (*C*). Results shown are mean ± SE of three independent experiments.

scriptional activity. Although the exact mechanism by which LR-90 modulates NF-κB activation is still unclear, our recent data suggest that LR-90's antioxidant properties may be partly responsible for the inhibition of NF-κB activation. NF-κB is a redox-sensitive transcription factor, and LR-90 exhibits both metal chelation and free radical scavenging activities (20) that could directly impact NF-κB activation. Indeed, ROS generation may be a common denominator in all the signaling pathways that lead to IκB degradation and NF-κB nuclear accumulation, as several studies demonstrated that various antioxidants, as well as overexpression of antioxidant enzymes, could inhibit NF-κB activation (42,43). In addition, a recent study reported that intracellular iron plays a critical role in NF-κB signaling in activated macrophages, and iron chelation inhibited the activation of this transcription factor (44).

Whether LR-90 directly interacts with RAGE or competes with S100b or RAGE ligands to influence its effects remain to be investigated. However, we observed similar inhibitory effects of LR-90 on MCP-1 expression on high glucose– and PMA (phorbol myristic acid)-treated THP-1 cells (data not shown). Moreover, our current study

showed that LR-90 prevented NF-κB activation when stimulated with TNF-α, another non-RAGE ligand like PMA. Taken together, these observations suggest that the mechanism of action of LR-90 may be intracellular, probably via modulation of the NF-κB signaling pathway.

Our current study also addressed the functional impact of the above findings on monocyte adhesion to the endothelium in vitro. Monocyte adherence is a first step leading to the maturation of monocytes into macrophage (27). We have shown earlier that high glucose, AGEs, and S100b can stimulate monocyte adherence to both endothelial and smooth muscle cells (32,33). Our current experiments showed that LR-90 dose-dependently prevented the adhesion of monocytes to HUVECs, which is consistent with our data on expression of MCP-1 and IP-10, two major chemoattractants involved in monocyte recruitment to vascular cells (45,46). Although we did not study the expression of other genes known to be involved directly in the binding of monocytes to endothelial cells such as β_2-integrins, E and P-selectins, ICAM-1 (intercellular adhesion molecule-1), PECAM-1 (platelet/endothelial cell adhesion molecule-1), and other chemokines and related adhesion molecules, it is reasonable to speculate that LR-90 can also inhibit the expression of these molecules via modulation of RAGE expression and/or NF-κB activation based on the following studies: *1*) that RAGE serves as a ligand for β_2-integrin Mac-1 (47); *2*) that blocking of RAGE ligation by soluble RAGE prevented vascular cell adhesion molecule expression in endothelial cells (48); and *3*) that PECAM-1, ICAM, and E- and P-selectins are all transcriptionally regulated by NF-κB (42,49).

We also evaluated the possible cytotoxic effects of LR-90 on monocytes, since theoretically, these toxic effects could have been the cause for some of the effects observed in our experiments. We did not detect any cytotoxic effects on cells treated with LR-90, even at the highest dose tested (200 μmol/l). Moreover, LR-90 did not affect the expression of 18S mRNA and actin and histone H2B protein levels used as internal control standards in the RT-PCR and Western blotting experiments, respectively. These findings further confirmed that the effect of LR-90 was not mediated by cellular death or cytotoxity but rather by specific inhibition of expression and secretion of the proinflammatory molecules.

In conclusion, our study shows for the first time that the AGE inhibitor LR-90 also has anti-inflammatory effects on activated monocytes and thus may have dual beneficial effects in the treatment inflammatory diseases such as atherosclerosis and other diabetes complications.

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