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Mechanoregulation of Monocyte Chemoattractant Protein-1 Expression in Rat Vascular Smooth Muscle Cells

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ABSTRACT

The authors have previously shown that arterial wall strain mediates the development of vessel wall inflammation in experimental hypertension. The current studies explore the mechanoregulation of monocyte chemoattractant protein-1 (MCP-1), a potent pro-inflammatory chemokine, by mitogen-activated protein kinases (MAPK) and oxidative stress. Rat aortic smooth muscle (RASM) cells were subjected to cyclic strain on a uniform biaxial strain device. Strain rapidly activated both ERK1/2^{MAPK} and p38^{MAPK}, with peak activation at 5 min. Strain induced a twofold increase in MCP-1 mRNA, which was attenuated by PD 98059, a specific ERK1/2^{MAPK} inhibitor, and SB 203580, a specific p38^{MAPK} inhibitor. Cyclic strain also increased production of superoxide anion via an NADPH oxidase-dependent mechanism. To assess the potential role of reactive oxygen species in MAPK activation, cells were stretched in the presence of *N*-acetylcysteine, which had no effect on p38^{MAPK} activation, but significantly inhibited ERK1/2^{MAPK} activation and MCP-1 expression. In conclusion, redox-sensitive activation of ERK1/2^{MAPK} and redox-insensitive activation of p38^{MAPK} regulate strain-induced MCP-1 expression in RASM cells. These findings define a role for MAPK signal transduction in establishing a pro-inflammatory state in the arterial wall, and thus implicate a potential molecular link between arterial wall strain and atherosclerosis. *Antioxid. Redox Signal.* 8, 1461–1471.

INTRODUCTION

HEMODYNAMIC FACTORS PLAY a pivotal role in the function, growth, and pathophysiologic adaptations of the arterial wall (reviewed in Ref. 21). Both blood pressure and blood flow result in mechanical forces applied to the vessel wall. Generally, blood pressure can be thought to govern arterial wall strain, whereas blood flow governs shear forces. The precise contribution of increased wall strain, as in hypertension, to the development of vascular pathology remains to be elucidated. Ollerenshaw *et al*. demonstrated that in experimental hypertension, increased wall strain is an absolute requirement for the development of vascular hypertrophy (22). Similarly, Thubriker and colleagues suggested that pressure-induced arterial wall strain is responsible, in part, for the anatomic localization of atherosclerotic lesions (32). In sum, these studies imply an important role for arterial wall strain in the development of vascular disease.

Our laboratory recently has shown that in two different models of experimental hypertension, arterial expression of monocyte chemoattractant protein 1 (MCP-1) is dramatically upregulated (6). This response appears to be mediated by mechanical factors, as reducing blood pressure through pharmacologic means normalizes the response. In addition, we and others have shown that MCP-1 expression can be upregulated by cellular deformation (6, 35–37). Thus, it appears that biologically relevant changes in arterial wall strain can mediate proinflammatory responses in the arterial wall. The cellular and molecular mechanisms involved in this response have not yet been fully defined.

MCP-1 is a potent proinflammatory chemokine secreted by vascular smooth muscle cells (VSMC), endothelial cells, and

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monocytes. It has been detected in human and experimental atherosclerotic plaques, as well as in the hypertensive aorta, implying an important role for this chemokine in the pathogenesis of vascular lesion formation (6, 40, 41). Although MCP-1 expression in hypertensive vessels and atherosclerotic lesions has been well described, specific signal transduction pathways regulating its expression are not well understood. The MCP-1 promoter contains both NF-κB and AP-1 transcription factor binding sites (33, 36), both of which are activated by cyclic strain in endothelial cells(37). The NF-κB transcription factor is known to be redox-sensitive, and therefore raises the possibility of oxidative signal transduction in the regulation of MCP-1. Indeed, our laboratory and others have documented the generation of reactive oxygen species (ROS) by mechanical deformation of vascular endothelial (14, 15) and smooth muscle cells (14). The AP-1 transcription factor is known to be both redox-sensitive (37) and downstream of mitogen-activated protein kinases (MAPK) (10). MAPK mediate intracellular responses to extracellular signals, including ROS (3, 12, 16) and mechanical deformation (1, 4, 25, 27, 38, 39), and thus also represent a potential mechanism for the regulation of MCP-1 by strain. Furthermore, activation of MAPK by deformation of VSMC and cardiomyocytes has been described by a number of groups (1, 4, 25, 27, 38, 39).

MAPK comprise a family of serine/threonine kinases that can be grouped into at least three distinct classes: the extracellular signal-regulated kinases 1 and 2 (ERK1/2^{MAPK}, also identified as p44/42^{MAPK}), c-Jun NH_2-terminal kinase (JNK^{MAPK}, also identified as p54^{MAPK}), and p38^{MAPK}. ERK1/2^{MAPK} was the first MAPK to be characterized. Its activation by a number of growth factors (*i.e.*, basic fibroblast growth factor) proceeds via a highly conserved ras/raf/MEK cascade (reviewed in Ref. 9). JNK^{MAPK} and p38^{MAPK} were originally described to be activated by cellular stress (*i.e.*, inflammatory cytokines, osmotic shock, endotoxin, ischemia, ultraviolet radiation), and thus were termed stress-activated protein kinases (SAPK). However, p38^{MAPK} activation also occurs in response to angiotensin II stimulation (34), as well as other stimuli. Like ERK1/2^{MAPK}, activation of JNK^{MAPK} and p38^{MAPK} proceeds through a well-conserved three-tiered kinase cascade.

In the present study, we demonstrate that MCP-1 upregulation by mechanical strain occurs via activation of a redox-sensitive ERK1/2^{MAPK} and a redox-insensitive p38^{MAPK}. These findings suggest a prominent role for MAPK signal transduction pathways in establishing a pro-inflammatory state of the arterial wall, and define cellular mechanisms that may provide a functional link between arterial wall strain and atherosclerosis.

METHODS

Materials and reagents

SB 203580 and PD 98059 were purchased from Calbiochem-Novabiochem Corp. (La Jolla, CA). Silicone sheeting (0.005 in) was purchased from Specialty Manufacturing (Saginaw, MI) and human plasma fibronectin from Life Technologies (Gaithersberg, MD). TRI reagent was purchased from Molecular Research Center (Cincinnati, OH), [^{32}P]-dCTP was from DuPont NEN (Boston, MA). "Complete Mini" protease inhibitor was from Boehringer Mannheim (Indianapolis, IN) and Magna NT nylon membranes were from Micron Separation, Inc. (Westboro, MA). QuickHyb hybridization solution and Prime-it II probe labeling kits were purchased from Stratagene (La Jolla, CA). Antibodies to total ERK1/2^{MAPK} and p38^{MAPK} were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), antibodies to phosphorylated ERK1/2^{MAPK} and phosphorylated p38^{MAPK} (Thr180/Tyr182) were from New England Biolabs (Beverly, MA). The phosphorylation-specific antibodies detect only MAPK that have been activated by phosphorylation on TXY. Detection of MAPK activity with these antibodies correlates well with direct measures of MAPK activity (42). Goat anti-rabbit horseradish peroxidase-conjugated secondary antibody, Tween, Biospin P50 columns and protein assay materials were purchased from Bio Rad Laboratories (Hercules, CA). Enhanced chemiluminescence western blotting detection system and mouse anti-rabbit antibody were from Amersham Life Science Corp. (Arlington Heights., IL). Calf serum was from Gibco BRL (Gaithersburg, MD). Fetal Bovine Serum was from Atlanta Biologicals (Norcross, GA). DMEM and all other chemicals and were purchased from Sigma Chemical Co. (St. Louis, MO).

Cell culture and strain experiments

Rat aortic smooth muscle (RASM) cells were isolated from the thoracic aortas of rats, as previously described (6). Cells were grown in DMEM supplemented with 4500 mg/l D-glucose, 2 m*M* L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 25 m*M* HEPES, and 10% heat-inactivated calf serum. Cells between passages 3 and 15 were plated onto flexible fibronectin-coated silicone membranes and fed every other day until near confluent. Plates were then washed twice with serum-free DMEM, and quiesced in 0.1% serum DMEM for 72 h. The quiesced cells were subjected to cyclic stretch at using a strain unit housed in an incubator at 37°C with 5% CO_2. Transgenic cell lines that overexpress human catalase (pCI-neo/catalase) or vector alone (pCI-neo) were maintained in 10% fetal bovine serum with 400 μg/ml geneticin.(42) Once plated onto fibronectin-coated silicone membranes for stretch experiments, these cells were grown in 10% calf serum, as described above. In this line of RASM cells, both catalase mRNA and protein are increased and the functionality of these cells have been demonstrated in studies showing an inhibition of Ang II-induced cellular hypertrophy (42). All stretch experiments were performed on a strain device developed by M.L. Gray and produced by Z-Development (Arlington, MA) (29). A finite element analysis performed by Brown and co-workers (5) indicates that this device provides uniform biaxial strain across the membrane surface with negligible fluid shear forces over the majority of the culture surface.

Protein extraction and Western blot analysis

For the MAP kinase assays, dishes were washed three times with cold phosphate buffered saline and cells were

lysed with a solution containing 50 m*M* HEPES, 5 m*M* EDTA, 50 m*M* NaCl, and 1% Triton. The lysis buffer also contained "Complete Mini" protease inhibitor and 50 m*M* NaF, 1 m*M* Na_3VO_4, and 10 m*M* $Na_4P_2O_7$ for phosphatase inhibition. Cells were suspended and transferred to microcentrifuge tubes, incubated at 4°C for 60 min, and centrifuged at 12,000 *g* for 10 min. For MAPK Western blot analysis, 15 μg of protein per sample was loaded onto a 12% polyacrylamide minigel and size fractionated. Total protein was transferred to a nylon membrane and blocked with 5% powdered milk, 0.5% Tween for 60 min. Incubation with primary antibodies was for 60 min each, followed by three 5 min washes in 0.2% milk, 0.2% Tween. Western analyses of MAPK activation employed phosphorylation-specific primary antibodies. Incubation with secondary antibodies was for 30 min, followed by three 5 min washes in 0.2% milk, 0.2% Tween. Membranes were then exposed to enhanced chemiluminescence solution for 1 min and exposed to radiograph film for 1–10 min.

RNA extraction, Northern blot analysis, and hybridization

Total RNA extraction was achieved by the guanidium thiocyanate method with phenol–chloroform extraction using Tri-Reagent. Fifteen micrograms of total RNA per sample was loaded onto a formaldehyde-containing 1% agarose gel. After size fractionation on a denaturing agarose–formaldehyde gel, total RNA was transferred to a nylon membrane and exposed to ultraviolet light to crosslink the RNA. Membranes were prehybridized for 20 min at 60°C with QuikHyb hybridization solution, and hybridized for 2 h with a random-primed [^{32}P]dCTP-labeled rat cDNA probe specific for MCP-1 (6). Membranes were washed twice for 15 min in 2X standard saline/citrate, 0.1% SDS at room temperature, and once in 0.2X standard saline/citrate, 0.1% SDS at 60°C for 30 min, then exposed to radiograph film at −70°C for 2–48 h. Laser densitometry and digital analysis of scanned images were used for radiograph quantitation, with all bands normalized to 28s rRNA.

Dihydroethidium staining for superoxide detection

Dihydroethidium (DHE) was purchased from Molecular Probes (Eugene, OR). DHE is permeable to cells, and it is specifically oxidized by intracellular superoxide to ethidium, which intercalates into the DNA in the nucleus (excitation/emission maxima 520/610 nm). DHE from working aliquots diluted in DMSO and sparged with N_2, was added in the dark at a final concentration of 10 μ*M* in colorless HBSS during the last 5 min of the strain application at both stretched dishes and static controls. Subsequently, cells were removed from the membranes as described previously, were placed on ice, and examined in a FACsort flow cytometer (Becton Dickinson, San Jose, CA) with a 488 nm excitation laser and absorption filter set at 575 ± 20 nm. A dish without DHE staining was used as negative control. Static and stretched cells were first examined under the microscope and subsequently a homogenous cell population was selected from a forward and side scatter box plot. Because of the logarithmic acquisition of the data, the geometric mean of the distribution in ethidium fluorescence intensity of 10,000 cells per sample was taken as an index of ethidium fluorescence and consequently of superoxide production.

Infection of cells with Nox1 antisense adenovirus

Subconfluent cells were cultured on stretch silicon membranes, counted in a Coulter Counter (Beckman Coulter Inc., Fullerton, CA) and infected overnight in serum free DMEM using a multiplicity of infection (MOI) of 10 with either the control vector (AdGFP), containing the green fluorescent protein (GFP) gene or the antisense nox1 virus (AdASnox1), that blocks the nox-1 homologue of gp21^{phox} of the NAD(P)H oxidase. The adenovirus was prepared from the pAdTrack-CMV vector at Dr. K.K. Griendling's laboratory (Emory University, Atlanta, GA). After the overnight incubation with the adenovirus, media were removed and cells were incubated in fresh media for another 48 h before the strain application. The infection efficiency 72 h after infection was >60%, as it was observed by the expression of GFP by means of fluorescence microscopy and flow cytometry. Cells were then stained with 10 μ*M* DHE for 5 min during strain or in static conditions, trypsinized, placed on ice, and analyzed by flow cytometric double-staining analysis.

Statistical analyses

All experiments were done at least three times. All data are presented as the mean ± SEM. An unpaired two-tailed Student's *t* test was used to analyze differences between two groups. Analysis of variance (ANOVA) with the Duncan new multiple range post hoc analysis was performed when more than two groups were compared. A p value of < 0.05 was considered significant.

RESULTS

Cyclic strain activates ERK1/2^{MAPK} and p38^{MAPK}

RASM cells subjected to cyclic strain had a normal appearance by phase-contrast microscopy. Strain did not induce cell loss as assessed by microscopy and protein determinations. To assess the effect of cellular deformation on MAPK in vascular smooth muscle, RASM cells grown on fibronectin-coated silicone membranes were subjected to 20% cyclic strain at 1 Hz. Compared with nonstretched control cells, stretched RASM cells exhibited activation of ERK1/2^{MAPK} and p38^{MAPK}, with peak activation at 5 min, as measured by Western blot analysis using phospho-specific anti-ERK1/2^{MAPK} and anti-p38^{MAPK} antibodies. There was no significant change in ERK1/2^{MAPK} or p38^{MAPK} total protein (Figs. 1 and 2). Densitometric quantification revealed an 8.5 ± 0.9-fold ($p < 0.0001$) increase in ERK1/2^{MAPK} phosphorylation and a 3.0 ± 0.6-fold ($p < 0.03$) increase in p38^{MAPK} phosphorylation (Figs. 1 and 2). Both ERK1/2^{MAPK} and p38^{MAPK} activation by cyclic strain occurred in a dose-dependent fashion over the range of 5–20% deformation (Fig. 3). These data reveal that both ERK1/2^{MAPK} and p38^{MAPK} are significantly activated by mechanical deformation of vascular smooth muscle.

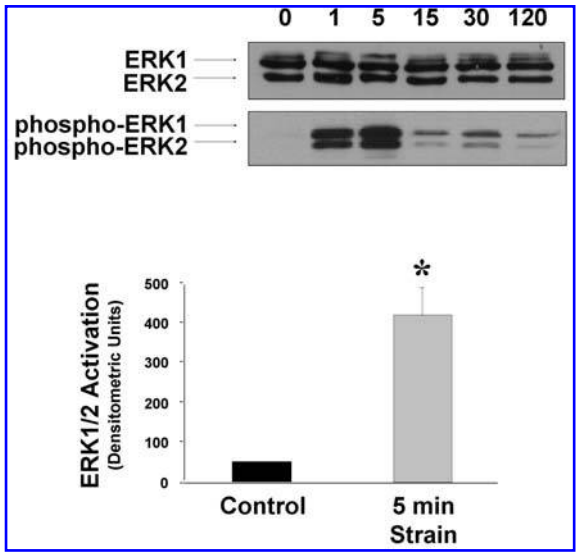

FIG. 1. Time-course for activation of ERK1/2^{MAPK} by cyclic strain. Western blot analysis using antibodies for total ERK1/2^{MAPK} and phosphorylated ERK1/2^{MAPK} was performed on protein harvested from RASM cells exposed to no strain or 20% cyclic strain at 1 Hz for 1, 5, 15, 30, or 120 min. A representative blot is shown, with densitometric quantification of six independent experiments below. * denotes p value < 0.0001 vs. control (Student's t test).

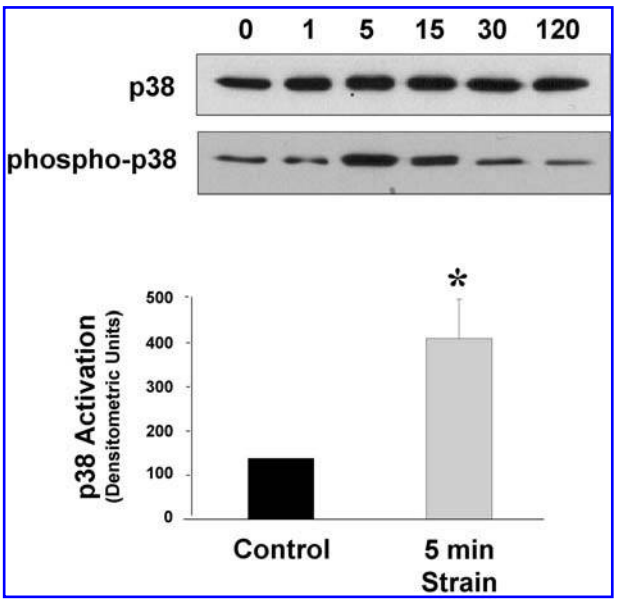

FIG. 2. Time-course for activation of p38^{MAPK} by cyclic strain. Western blot analysis using antibodies for total p38^{MAPK} and phosphorylated p38^{MAPK} was performed on protein harvested from quiesced RASM cells exposed to no strain or 20% cyclic strain at 1 Hz for 1, 5, 15, 30, or 120 min. A representative blot is shown, with densitometric quantification from six independent experiments below. * denotes p value < 0.03 vs. control (Student's t test).

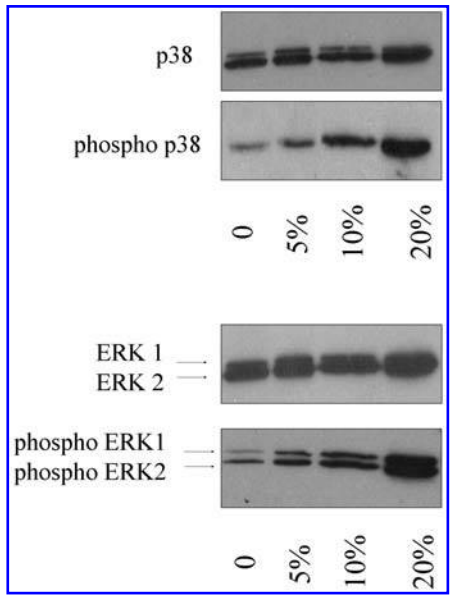

FIG. 3. Effect of different strain magnitudes on ERK1/2^{MAPK} and p38^{MAPK} activation. Representative Western blots for the phosphorylated forms of ERK1/2^{MAPK} and p38^{MAPK} in vascular smooth muscle cells exposed to a range of deformation from 5% to 20% for 5 min at 1 Hz. These data are representative of three such experiments.

Induction of MCP-1 by cyclic strain is dependent on ERK1/2^{MAPK} and p38^{MAPK}

Following the observation of ERK1/2^{MAPK} and p38^{MAPK} activation by cyclic strain (Figs. 1 and 2), and realizing that binding sites for potential downstream transcription factors NFkB and AP-1 are present in the MCP-1 promoter (33, 36), experiments were done to explore the dependency of MCP-1 induction on MAPK regulatory pathways. For these experiments, specific inhibitors of ERK1/2^{MAPK} and p38^{MAPK} were used. Stretch-induced ERK1/2^{MAPK} phosphorylation was attenuated 94% by 100 μM PD 98059. No change in ERK1/2^{MAPK} or p38^{MAPK} phosphorylation was seen with DMSO (1 μl/ml), which was used as a vehicle for the inhibitors. There was no demonstrable cross reactivity of the MAPK inhibitors at the concentrations used as there was no evidence of decreased phosphorylation of ERK1/2^{MAPK} by SB 203580, nor was there any significant decrease in phosphorylation of p38^{MAPK} by PD 98059.

RASM cells subjected to 20% cyclic stretch at 1 Hz exhibited a 2.0 ± 0.37-fold (p < 0.006) increase in MCP-1 mRNA at 2 h (Fig. 4). As shown in Fig. 4, this increase was attenuated 84% by specific inhibition of the ERK1/2^{MAPK} pathway with 100 μM PD 98059 (p < 0.02) and 71% by specific inhibition of the p38^{MAPK} pathway with 10 μM SB 203580 (p < 0.02). In both cases, the inhibitors reduced expression of MCP-1 to a level that was not significantly different from control, indicating essentially complete inhibition. MCP-1 mRNA levels were not affected by the

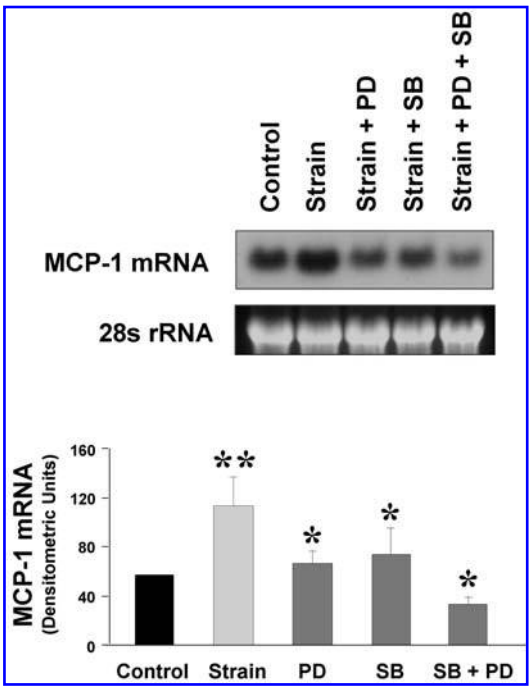

FIG. 4. Induction of MCP-1 by cyclic strain is dependent on both ERK1/2^{MAPK} and p38^{MAPK}. Northern blot analysis using a cDNA probe specific for MCP-1 mRNA was performed on RNA harvested from quiesced RASM cells exposed to no strain or 20% cyclic strain at 1 Hz for 2 h. The *third lane* represents RNA from stretched cells pretreated for 30 min with the ERK1/2^{MAPK} inhibitor PD 98059 (100 μ*M*). The *fourth lane* represents RNA from stretched cells pretreated for 30 min with the p38^{MAPK} inhibitor SB 203580 (10 μ*M*). The *fifth lane* represents RNA from stretched cells simultaneously pretreated for 30 min with both PD 98059 and SB 203580. A representative blot is shown, with densitometric quantification from three independent experiments below. Ethidium bromide staining of 28s rRNA from each sample is shown for comparison of loading.* denotes p value < 0.02 vs. 2 h stretch alone (ANOVA).** denotes p value < 0.006 vs. control (ANOVA). PD, PD 98059, SB, SB 203580.

presence of DMSO (1 μl/ml) alone, and there was no effect of the inhibitors on the baseline expression of MCP-1. These data suggest that cell deformation-induced MCP-1 is under the dual regulatory influence of both ERK1/2^{MAPK} and p38^{MAPK}.

Effects of mechanical strain on superoxide production

We first investigated the initial temporal activation of superoxide production due to cyclic strain in VSMC. As shown in Fig. 4, a physiologically relevant cyclic strain of 10% at 1 Hz applied for 5 min induced an increase in superoxide compared to the static cells. The time course of the differences between stretched and static cells is shown in Fig. 5. The mean absolute values in ethidium fluorescence intensity increased significantly from 19.3 ± 3.8 U at the static cells to 26.3 ± 3.9 U at 5 min (36% increase vs. static cells (p < 0.001). After 30 min of strain, superoxide generation decreased to 24.4 ± 3.9 U, (26% increase vs. static, p < 0.001) and further decreased at 1 h of strain, but remained significant higher compared to static cells (23.87 ± 3.39 U, 24% increase vs. static, p < 0.001).

To investigate the role of cyclic strain magnitude on superoxide production, different strain amplitudes over the range of 5–30% were applied for 5 min at 1 Hz. All strain amplitudes resulted in increased superoxide production compared to static cells (Fig. 6, p < 0.05). The maximal response occurred at 10% strain, which was not different from higher levels of strain.

We also evaluated the frequency response of the SMC to cyclic strain at 5 min of 10% strain. The absolute values of ethidium fluorescence with DHE staining show a significant increase at the frequencies of 0.5 Hz (19% increase vs. static cells, p < 0.05,1 Hz (24% increase vs. static cells, p < 0.05) and 2 Hz (27% increase vs. static cells, p < 0.05). The superoxide production at 0.05 Hz and 0.1 Hz was not statistically different from the static controls.

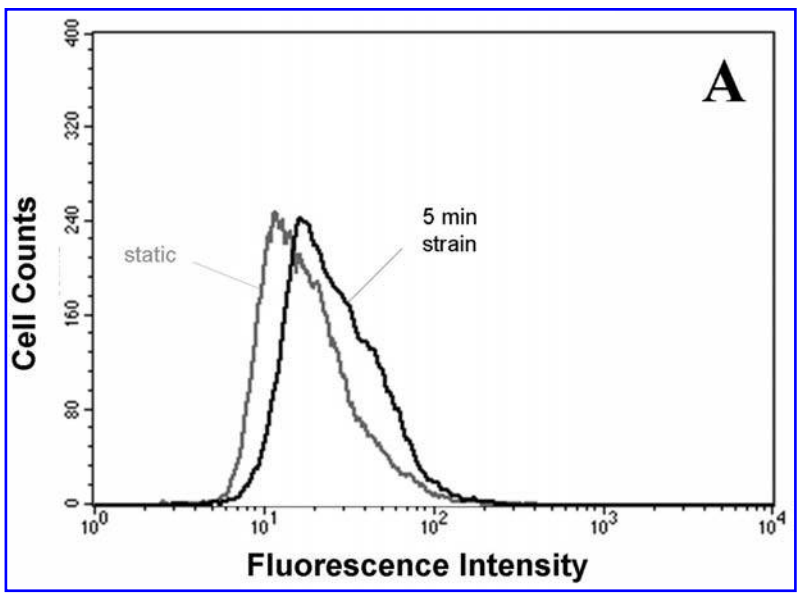

FIG. 5. Strain-induced superoxide production in VSMC. Cells were cultured on flexible silicon membranes and exposed to 10% cyclic strain for 5 min at 1 Hz or to static conditions. Cells were incubated with 10 μM DHE during strain or in static conditions for 5 min, removed by trypsinization, and analyzed by flow cytometry. The shift of ethidium fluorescence to the right by the stretched cells indicates increased intracellular superoxide production compared to static cells. Shown are added histograms from n = 4 independent experiments (number of cells per histogram are 33504 and 35289 cells for static and 5 min strain, respectively).

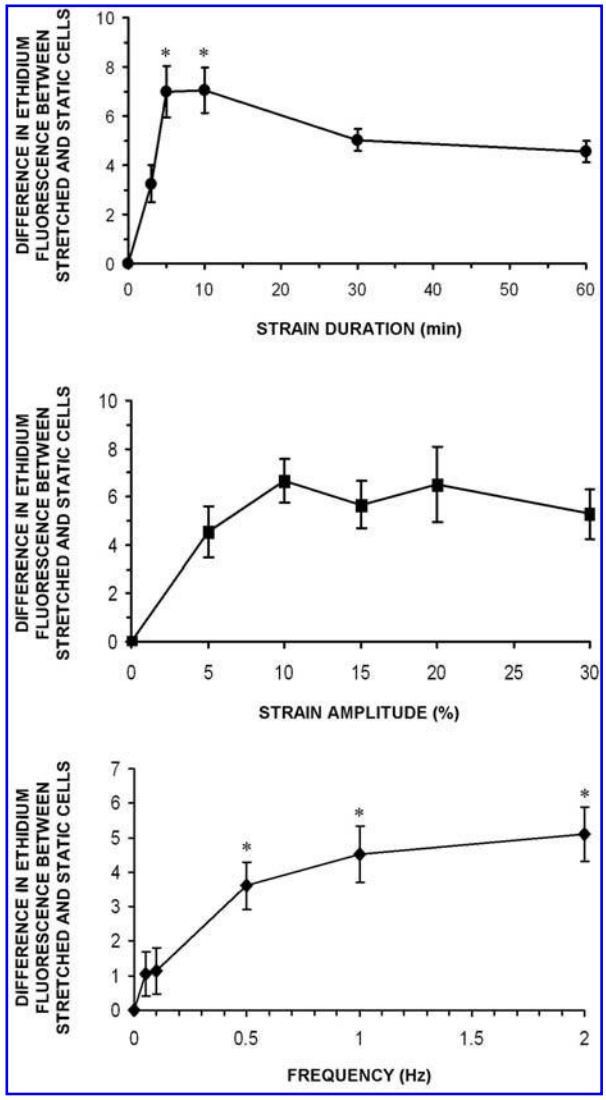

FIG. 6. Effects of mechanical stimuli on superoxide production in VSMC. Cells were exposed to cyclic strain and incubated with 10 μ*M* DHE for 5 min during strain or in static conditions. Shown are the differences in ethidium fluorescence intensity between stretched and matched static control cells accessed by flow cytometry. Zero time points indicate static controls. Data are shown as mean ± SEM. The *top panel* presents the time course of strain-induced superoxide production in VSMC after application of different durations of 10% cyclic strain at 1 Hz. The *middle panel* shows the effect of different strain amplitudes on superoxide production in VSMC after application of cyclic strain for 5 min at 1 Hz, The *bottom panel* depicts the effects of strain frequency on strain-induced superoxide production in VSMC. VSMC were exposed to cyclic strain for 5 min at the frequencies indicated or to static conditions. * = $p < 0.05$.

Inhibition of strain-induced superoxide by antisense nox-1 mRNA adenovirus

To examine the source of superoxide generation induced by cyclic strain in VSMC, we infected the cells with an adenovirus expressing antisense nox-1 mRNA. Figure 7 shows that strain-induced superoxide production after 10% strain for 5 min was significantly inhibited in the nox-1 antisense infected cells compared to the cells infected with the empty control vector (67% decrease vs. vector, $n = 4$, $p < 0.05$).

Cyclic strain-induced activation of ERK1/2^{MAPK} is redox-sensitive, while p38^{MAPK} activation is redox-insensitive

In addition to the data presented above, several groups, including our own, have demonstrated the generation of ROS by cyclic strain in vascular cells (14, 15). It is also clear that MAPK activation is redox sensitive in a number of experimental systems (12, 16, 34). Therefore, to assess the potential role of ROS in mediating MAPK activation by cell deformation, cells were treated with *N*-acetylcysteine, a potent antioxidant. Activation of ERK1/2^{MAPK} by 20% cyclic stretch at 1 Hz was diminished by 33% (± 7%, $p < 0.003$) when cells were pretreated for 30 min with 20 m*M* *N*-acetylcysteine, whereas activation of p38^{MAPK} was not affected (Fig. 8). To provide insights to the causative ROS, a similar set of experiments was done using RASM cells that overexpress the human catalase gene (34). No differences were seen in the strain-induced phosphorylation of ERK1/2^{MAPK} or p38^{MAPK} in cells overexpressing the catalase gene versus cells expressing vector only (Fig. 9). In sum, these results demonstrate that, in response to cell deformation, ERK1/2^{MAPK} is redox-sensitive, while p38^{MAPK} is redox-insensitive. Unlike the findings with angiotensin II-mediated activation of p38^{MAPK}, activation of ERK1/2^{MAPK} does not appear to be mediated by H_2O_2.

Induction of MCP-1 by cyclic strain is redox-sensitive

Following the observation that cyclic strain-induced MCP-1 is regulated by a redox-sensitive ERK1/2^{MAPK}, we explored the role of ROS in strain-induced MCP-1 expression. Compared to untreated stretched RASM cells, cells pretreated for 30 min with 20 m*M* *N*-acetylcysteine showed a 75 ±17% ($p < 0.02$) reduction in the upregulation of MCP-1 (Fig. 10).

DISCUSSION

Hypertension is a major risk factor for atherosclerotic vascular disease. The cellular and molecular mechanisms by which hypertension predisposes to atherosclerosis have proven elusive, but likely involve both circulating factors and alterations in arterial wall strain (30). Alterations in arterial wall strain also occur in normotensive individuals and, similarly, are associated with sites in the arterial tree that are sensitive to the early formation of atherosclerotic lesions (31, 32). MCP-1 is a potent chemokine secreted by vascular smooth muscle, endothelium, and monocytes/macrophages, which act to recruit circulating monocytes to the arterial wall. The migration of these inflammatory monocytes into the vessel wall is essential to fatty streak formation and atherogenesis (8). The data presented here provide support for the con-

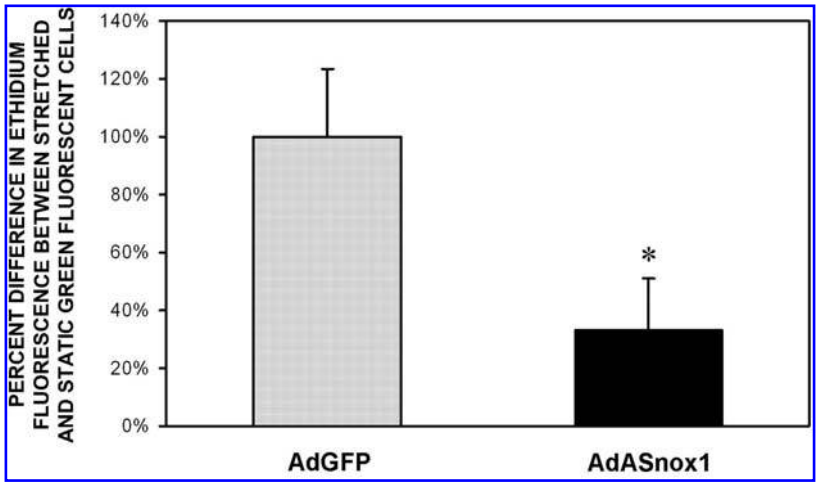

FIG. 7. Enzymatic source of strain-induced superoxide production in VSMC. Cells were infected with control vector (AdGFP) containing only the GFP gene or the antisense nox-1 mRNA adenovirus (AdASnox1), and then exposed to 10% cyclic strain for 5 min at 1 Hz. The difference in ethidium fluorescence between stretched and static cells that express green fluorescence shows that strain-induced superoxide production was significantly blocked by the cells infected with the nox-1 antisense adenovirus compared to control vector cells ($n = 4$, $p < 0.05$ vs. control vector).

cept that strain-mediated inflammatory responses in the arterial contribute to the pathogenesis of hypertensive and atherosclerotic vascular disease.

The important new findings presented here demonstrate that mechanical regulation of MCP-1 expression in vascular smooth muscle cells occurs via a redox-sensitive activation of ERK1/2^{MAPK} and a redox-insensitive activation p38^{MAPK}. Furthermore, we have shown that the increase in ERK1/2^{MAPK} and p38^{MAPK} activation occur over a range of physiologically relevant levels of strain. These findings support a prominent role for MAPK signal transduction pathways in establishing a pro-inflammatory state of the arterial wall, and suggest a mechanism by which alterations in wall stress accelerate the process of atherosclerosis.

MCP-1 is a potent chemokine that facilitates the migration of monocytes into the subintimal space, where they differentiate into macrophages, scavenge oxidized lipid, and may ultimately form foam cells and a fatty streak. Previous reports have documented increased MCP-1 expression in human and experimental atherosclerotic plaques (40, 41), with the primary source of MCP-1 in these models appearing to be VSMC and macrophages (41), although some activity may also arise from the endothelium (36, 37). Corroborative cell culture experiments suggest that MCP-1 accounts for a majority of the monocytic chemotactic activity produced by VSMC (24). Thus, vascular inflammation, regulated by the induction of MCP-1 expression in concert with adhesion molecules represents a possible molecular mechanism for accelerated atherosclerosis associated with hypertension (reviewed in Refs. 2 and 7). It is interesting to note that another major risk factor for the development of atherosclerosis, hypercholesterolemia, is also associated with induction of vascular wall MCP-1 and inflammation (41). Together, these studies suggest that the induction of MCP-1 and the subsequent development of vessel wall inflammation represent an early step in the pathogenesis of vascular lesions, and may be a common molecular mechanism for a number of risk factors for atherosclerosis.

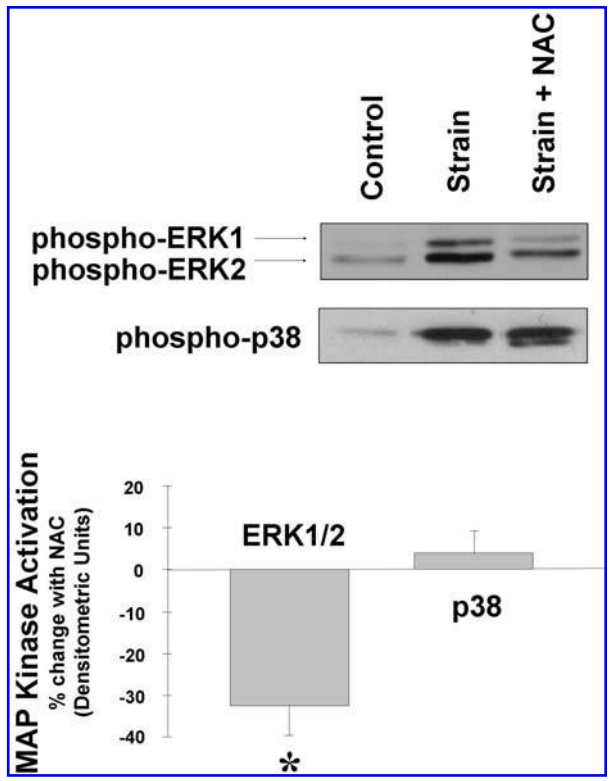

FIG. 8. Cyclic strain-induced activation of ERK1/2^{MAPK}, but not p38^{MAPK}, is inhibited by *N*-acetylcysteine. Western blot analyses using an antibody specific for phosphorylated ERK1/2^{MAPK} (*upper panel*) or phosphorylated p38^{MAPK} (*lower panel*) was performed on total protein harvested from quiesced RASM exposed to no strain or 20% cyclic strain at 1 Hz for 5 min. The *third lane* represents protein from stretched cells pretreated for 30 min with 20 m*M N*-acetylcysteine. A representative blot is shown, with densitometric quantification from three independent experiments below. Densitometry depicts the change in activated ERK1/2^{MAPK} or p38^{MAPK} in cells stretched in the presence or absence of *N*-acetylcysteine. NAC, *N*-acetylcysteine.

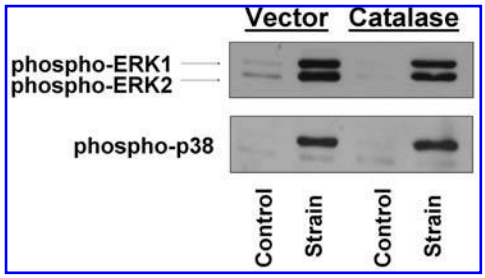

FIG. 9. Cyclic strain-induced activation of ERK1/2^{MAPK} and p38^{MAPK} is not regulated by H_2O_2. Western blot analyses using an antibody specific for phosphorylated ERK1/2^{MAPK} (*upper panel*) or phosphorylated p38^{MAPK} (*lower panel*) was performed on total protein harvested from quiesced RASM cells that overexpress human catalase (pCI-neo/catalase) or vector alone (pCI-neo) following exposure to no strain or 20% cyclic strain at 1 Hz for 5 min. A representative blot from three independent experiments is shown.

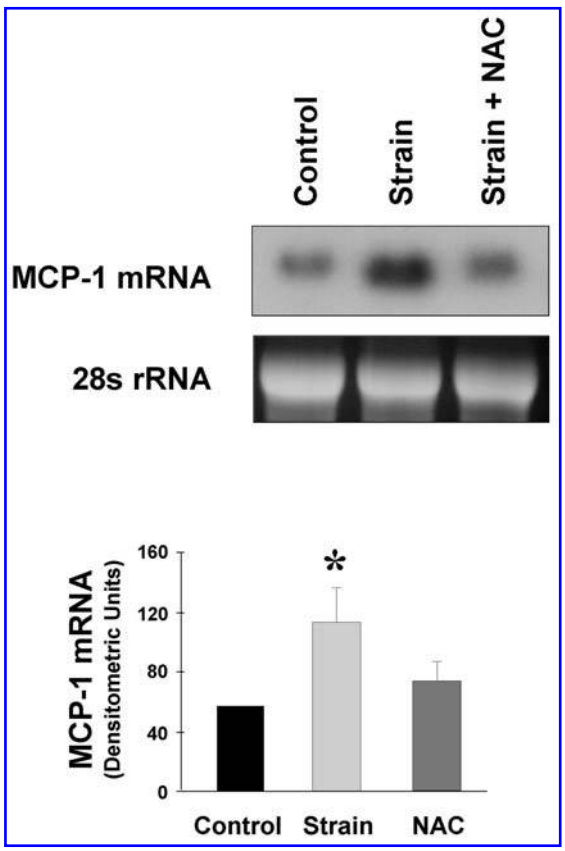

FIG. 10. Induction of MCP-1 by cyclic strain is inhibited by *N*-acetylcysteine. Northern blot analysis using a cDNA probe specific for MCP-1 mRNA was performed on RNA harvested from quiesced RASM cells exposed to no strain or 20% cyclic strain at 1 Hz for 2 h. The *third lane* represents RNA from stretched cells pretreated for 30 min with 20 m*M N*-acetylcysteine. A representative blot is shown, with densitometric quantification from three independent experiments below. Ethidium bromide staining of 28s rRNA from each sample is shown for comparison of loading. * denotes p value < 0.02 vs. 2 h stretch alone. NAC, *N*-acetylcysteine.

It has been previously shown that deformation of vascular smooth muscle cells by cyclic strain activates both the ERK1/2^{MAPK} (1, 4, 25, 27, 38, 39) and p38^{MAPK} (18, 19) pathways. It is interesting that in response to cyclic strain, ERK1/2^{MAPK} activation is redox-sensitive, while p38^{MAPK} activation is redox-insensitive. This is in contrast to MAPK activation by angiotensin II, in which p38^{MAPK} is redox-sensitive and ERK1/2^{MAPK} is redox-insensitive (34). These data raise the possibility that a single pathophysiologic condition like angiotensin II-mediated hypertension may have synergistic signaling mechanisms in VSMC for regulating the earliest steps in the development of arterial wall inflammation. In addition, these data demonstrate that ERK1/2^{MAPK} and p38^{MAPK} work in concert to upregulate MCP-1 expression in response to vascular smooth muscle deformation. Both appear necessary but not sufficient for strain-induced upregulation of MCP-1.

The demonstration that ERK1/2^{MAPK} activation by cell deformation is redox-sensitive provides insight to the fundamental importance of ROS generation in vascular inflammation. Data from several groups demonstrate that ROS are necessary for the induction of inflammatory mediators, in particular, vascular cell adhesion molecule-1 and MCP-1 (20, 28). Our data provide evidence that a proximal, redox-sensitive step in MCP-1 regulation involves ERK1/2^{MAPK}. The responsible ROS is as yet undetermined, but based on the lack of effect of catalase on strain-induced ERK1/2^{MAPK} activation, we hypothesize that a reactive oxygen species other than H_2O_2, (perhaps superoxide anion) is the likely causative species. Indeed, it has been demonstrated that ERK1/2^{MAPK} is preferentially activated by superoxide anion (3). This is further supported by evidence that superoxide production, NADPH oxidase activity, and NADPH oxidase p22 phox subunit expression are upregulated in experimental hypertension (11, 17, 26). It is of interest to note that while there was a clear dose-response relationship between strain magnitude and MAPK activation, a similar response was not seen in superoxide production. This finding is in apparent conflict with our conclusion linking reactive oxygen species and p38^{MAPK} activation. One very likely explanation for these results is that there is convergence of several different pathways that regulate gene expression. Indeed, our data demonstrate that MCP-1 expression is regulated by parallel signaling pathways that are conceptually similar.

The relationship between mechanical devices that expose cultured cells to mechanical strain and the actual mechanical environment *in vivo* is currently difficult to define. There have been multiple efforts made to model the mechanical forces that occur within the arterial wall (*e.g.*, Refs. 31 and 32). A major strength of these models is that they permit analyses of the distribution of wall stress in relatively complex geometries. However, they are limiting in their abilities to predict the stresses to which individual cells are exposed. More direct measurements of vessel deformation have shown that epicardial coronary arteries distend from 10 to 22% during the cardiac cycle. Similarly, the circumference of the carotid artery increases by up 10% during the normal cardiac cycle. These changes would be higher under conditions of increased pulse pressure (reviewed in Ref. 23). Similar magni-

tudes of wall strain have been reported in the mesenteric arteries (13). Thus, the range of cyclic strain applied to the cultured cells in these studies encompasses the estimated degree of deformation observed *in vivo*.

There are several aspects of the current work that deserve further comment. The first is the selection of the stretch device that was used for these studies. A number of devices have been designed to attempt to apply a uniform mechanical strain to cultured cells. The careful design and operation of these devices is of critical importance in carrying out cell-deformation experiments as significant theoretical and practical mechanical problems exist (29). Analyses of some devices suggest that the strain profile across the cell culture surface is nonuniform, resulting in heterogeneous cell stretch and even some degree of cell compression. (5) In addition, significant fluid shear forces can occur in some systems. Given that shear stress is a potent activator of MAP kinase signaling, the selection of the device used to expose cells to mechanical strain is of critical importance. For the studies presented here, we employed a device with a cam-driven piston applying upward deformation to a flexible silicone membrane. This device has proven to result in remarkably uniform biaxial strain with negligible fluid shear forces (5, 29). There are other factors, such as the coating of the culture surface, that can modulate the cellular responses to strain (27). These differences may help to explain some of the discrepancies between our work and that of others. For example, Li *et al*. demonstrated that JNK activation occurred in smooth muscle cells exposed to cyclic strain (18, 19), a finding that we could not substantiate (data not shown). It is likely that some or all of the factors above contributed to these differences. Ultimately, validation of the importance of these signaling mechanisms requires confirmation *in vivo.*

In summary, this study demonstrates that cyclic deformation of VSMC results inproduction of superoxide and subsequent redox-sensitive activation of ERK1/2^{MAPK} as well as redox-insensitive activation of p38^{MAPK}. Both pathways act in concert to regulate MCP-1 expression, suggesting a prominent role for MAPK signal transduction in establishing a proinflammatory state in the arterial wall. These findings provide a potential mechanistic link between arterial wall strain and vascular pathology.

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ABBREVIATIONS

ERK1/2^{MAPK}, extracellular signal regulated mitogen-activated protein kinase 1/2; JNK^{MAPK}, c-Jun NH_2-terminal mitogen-activated protein kinase; MAPK, mitogen-activated protein kinase; MCP-1, monocyte chemoattractant protein-1; RASM, rat aortic smooth muscle; ROS, reactive oxygen species; VSMC, vascular smooth muscle cell.

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