Acrolein Induces Cyclooxygenase-2 and Prostaglandin Production in Human Umbilical Vein Endothelial Cells

Roles of p38 MAP Kinase

Yong Seek Park, Jayoung Kim, Yoshiko Misonou, Rina Takamiya, Motoko Takahashi, Michael R. Freeman, Naoyuki Taniguchi

Objective—Acrolein, a known toxin in tobacco smoke, might be involved in atherogenesis. This study examined the effect of acrolein on expression of cyclooxygenase-2 (COX-2) and prostaglandin (PG) production in endothelial cells.

Methods and Results—Cyclooxygenase (COX)-2 induction by acrolein and signal pathways were measured using Western blots, Northern blots, immunoflourescence, ELISA, gene silencing, and promoter assay. Colocalization of COX2 and acrolein-adduct was determined by immunohistochemistry. Here we report that the levels of COX-2 mRNA and protein are increased in human umbilical vein endothelial cells (HUVECs) after acrolein exposure. COX-2 was found to colocalize with acrolein-lysine adducts in human atherosclerotic lesions. Inhibition of p38 MAPK activity abolished the induction of COX-2 protein and PGE2 accumulation by acrolein, while suppression of extracellular signal-regulated kinase (ERK) and JNK activity had no effect on the induction of COX-2 expression in experiments using inhibitors and siRNA. Furthermore, rotterlin, an inhibitor of protein kinase Cδ (PKCδ), abrogated the upregulation of COX-2 at both protein and mRNA levels.

Conclusion—These results provide that acrolein may play a role in progression of atherosclerosis and new information on the signaling pathways involved in COX-2 upregulation in response to acrolein and provide evidence that PKCδ and p38 MAPK are required for transcriptional activation of COX-2. (Arterioscler Thromb Vasc Biol. 2007;27:1319-1325.)

Key Words: acrolein ■ COX-2 ■ p38 MAPK ■ atherosclerosis ■ endothelial cells

Activation of endothelial cells by proinflammatory stimuli has been established as an important link between risk factors and the pathologic mechanisms underlying atherosclerosis.1 Thus, control of the inflammatory status of endothelial cells, which is achieved by a balance of pro- and antiinflammatory signals, is crucial to limiting the disease. Tobacco smoking induces inflammatory reactions2 and promotes atherosclerosis3; however, the mechanism that links cigarette smoking to an increased incidence of atherosclerosis is poorly understood.

Acrolein (CH$_3$=CH-CHO), a major product of organic combustion, including tobacco smoking, is the most reactive $\alpha$, $\beta$-unsaturated aldehyde found widely in the environment. Acrolein is highly reactive and is hazardous to human health.4 Acrolein is produced by a wide variety of both natural and synthetic processes, including the incomplete combustion of organic materials. Acrolein also has been found to be formed from threonine by neutrophil myeloperoxidase at sites of inflammation5 and has been identified as both a product and initiator of lipid peroxidation.6 Recent studies have shown that acrolein levels are increased in many diseases such as atherosclerosis, Alzheimer disease, and diabetes, and is possibly related to pathogenesis in these conditions.7–9 We and others have reported that acrolein elevates intracellular reactive oxygen species (ROS) levels, which leads to cell dysfunction.8,10 ROS-mediated cell damage is an important etiologic factor in the pathogenesis of atherosclerosis.11 ROS has been reported to induce the production of various atherogenic factors including inflammatory proteins.12

Cyclooxygenase (COX) catalyzes the oxygenation of arachidonic acid to prostaglandin (PG) endoperoxides, which are converted enzymatically into PGs and thromboxane A2, both of which play physiological as well as pathologic roles in vascular function. Two distinct isoforms of COX have been identified in mammalian cells. COX-1 is constitutively expressed in a variety of cells such as vascular cells, fibroblasts, platelets, and epithelia, whereas COX-2 is absent from most normal tissues but is expressed in response to proliferative and inflammatory stimuli.13 COX-2 is expressed in ath-

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erosclerotic lesions and is increased after vascular injury. Because chronic inflammation plays an important role in atherosclerosis, COX-2 may participate in the genesis of atherosclerosis. In view of the reports that inflammatory responses elicited by tobacco smoking are closely associated with atherogenesis, we hypothesized that acrolein, which is a main carbonyl component of tobacco smoke, might be involved in atherogenesis. In this report, we have explored the effect of acrolein on expression of COX-2 and PGE₂ production in endothelial cells. We showed (1) that acrolein induced COX-2 expression at both mRNA and protein levels, and (2) that this induction required the activation of PKCδ, p38 MAPK, and CREB. We also demonstrated (3) that treatment with p38 MAPK inhibitor reversed PGE₂ secretion by acrolein treatment, suggesting that the p38 MAPK pathway is important mechanistic component of this process.

Methods
The Methods are provided as supplemental online material, available at http://atvb.ahajournals.org.

Results
Acrolein Induces COX-2 Expression and PGE₂ Production
To test the effects of acrolein on HUVECs, cells were incubated with medium containing acrolein at different concentrations, and COX-2 levels were analyzed by Western blot. COX-2 was strongly induced in HUVECs in dose- and time-dependent manner (Figure 1A and 1B). Northern blotting analysis showed that the COX-2 mRNA signal was more intense in the cells that had been treated with acrolein, compared with control cells. The induction was detected at 0.5 hour after the addition of acrolein and reached the maximal level after 1 hour (Figure 1C). Immunofluorescence analysis demonstrated that treatment with 10 μmol/L acrolein in the presence or absence of NS-398 for 16 hours, and then release of PGE₂ was measured from supernatants as described in Methods. The values shown for PGE₂ production are the mean±SD of 3 independent experiments. *P<0.001 compared with untreated control cells. F, Immunoblot analysis of COX-2 in lung tissues of mice administrated acrolein (C: control, PBS only, A: acrolein 4 mg/Kg). Human atherosclerotic blood vessel specimens were immunostained with anti-acrolein antibody (G) and anti-COX-2 (H) as described in Methods. Bar=50 μm.
itor, NS-398. The results indicate that acrolein can lead to COX-2 protein expression and subsequently PGE₂ biosynthesis in HUVECs.

Induction of COX-2 by Administration of Acrolein in Mice and COX-2 Colocalizes With Protein-Bound Acrolein in Atherosclerotic Lesions From Patients

To further study whether acrolein is capable of inducing COX-2 in vivo, mice administered with acrolein (4 mg/kg) for 24 hours and we found COX-2 was induced by acrolein in lung tissues (Figure 1F). Next, we also examined the pathohistologic location of COX-2 and acrolein-bound protein in human atherosclerotic blood vessels samples to determine whether acrolein might be involved in COX-2 upregulation in vivo. Both COX-2 and protein-bound acrolein were rarely detected in nonatherosclerotic segments of these blood vessels (data not shown). In contrast, we found that both acrolein-lysine adducts (Figure 1G) and COX-2 (Figure 1H) colocalized in the blood vessel cells.

Inhibition of p38 MAPK Abolishes Induction of COX-2 Protein Expression

Oxidative stress triggered by H₂O₂ and treatment with lipid peroxidation end products have been found to activate MAP kinase pathways including ERK, JNK, and p38 MAPK.₁₆,₁₇ To test whether acrolein activates MAP kinase pathways including ERK, JNK, and p38 MAPK in HUVECs, cells were exposed to acrolein in the culture medium. We found activation of these kinases by acrolein (supplemental Figure I). To determine whether the MAPK pathways were directly involved in the induction of COX-2 by acrolein, cells were pretreated with kinase inhibitors for 1 hour before adding acrolein. PD98059, which specifically inhibits the ERK, had no effect on the induction of COX-2 (Figure 2A), suggesting that the induction of COX-2 did not require the ERK pathway. SP600125, JNK specific inhibitor,¹₈ also did not prevent the induction of COX-2 protein by acrolein. In contrast, SB203580, a specific inhibitor for p38 MAPK, potently inhibited the induction of COX-2. Interestingly, we observed that acrolein did not affect COX-1 expression (Figure 2A). To determine whether the inhibition occurred at the level of transcription, Northern blot analysis was carried out. The levels of COX-2 mRNA were dramatically reduced by SB203580 but not by PD98059 and SP600125 (Figure 2B). We next investigated the effect of MAPK inhibitors on acrolein induced PGE₂ production. The p38 MAPK inhibitor, SB203580, dramatically suppressed acrolein-induced PGE₂ production (Figure 2C), suggesting that p38 MAPK signaling pathway is involved in the acrolein-induced PGE₂ biosynthesis. But PD98059 and SP600125 did not prevent the PGE₂ production by acrolein.

To validate a role for p38 MAPK in the acrolein induced COX-2 upregulation, we inhibited p38 MAPK using siRNA transfection. Transfection of HUVECs with p38 MAPK siRNA duplex (250 nmol/L, 48 hours) abolished the induction of COX-2 by acrolein (Figure 3A). Figure 3B shows that acrolein stimulated the transcription of COX-2 as well. When we performed transient transfections with a human COX-2 promoter-luciferase reporter construct (−1432/+59),¹⁹ COX-2 reporter activity was increased to more than 100% by acrolein and decreased to basal levels by treatment of p38 MAPK inhibitor.

PKC Is Required for COX-2 Activation

Because our previous data indicated that acrolein activates PKCδ,₁₀ we examined the role of PKC in COX-2 induction by acrolein. Figure 4A shows that acrolein rapidly activates PKCδ. Within 10 minutes, PKC activation reached the maximal level, and the kinase activity gradually decreased at 30 minutes after stimulation. To determine whether PKCδ plays a role in the signaling pathways controlling the induction of COX-2 by acrolein, we used an inhibitor specific for
PKCδ, rottlerin. HUVECs were pretreated with 5 μmol/L concentrations of rottlerin for 1 hour, and then 10 μmol/L acrolein was added. After 16 hours, cells were collected, and the COX-2 protein levels were determined by Western blot analysis. Figure 4B shows that 5 μmol/L rottlerin completely blocked the induction of COX-2 protein. Northern blot analysis (Figure 4C) shows that the inhibition occurred at the level of mRNA, suggesting that the activation of a PKC kinase activity is necessary for the upregulation of the COX-2 mRNA in response to acrolein treatment. Taken together, these data support the conclusion that the activation of PKC is required for COX-2 induction.

Activation of p38 MAPK Is Affected by Rottlerin
To determine whether the p38 MAPK activation is followed by PKC activation in response to acrolein treatment, HUVECs were pretreated with rottlerin, then were incubated with 10 μmol/L acrolein. The activation of p38 MAPK was determined by Western blot analysis (Figure 4D). We found that a significant decrease of p38 MAPK in the presence of rottlerin, suggesting that PKCδ is required for the activation of p38 MAPK by acrolein treatment.

Phosphorylation of CREB and Activation of CRE Transcription Factor by Acrolein
We next investigated acrolein-associated phosphorylation of cAMP-responsive element-binding protein (CREB), which is known as a regulator of COX-2 expression in several cells. Acrolein strongly increased the phosphorylation of CREB in HUVECs (Figure 5A), and the activation was completely abolished by p38 MAPK inhibitor but not by ERK and JNK inhibitor (Figure 5A). The COX-2 promoter
were stimulated with 10 μmol/L acrolein and anti-CREB antibody. B, CRE COX-2 binding. Cells were subjected to Western blotting using anti–phospho-CREB antibody and anti-CREB antibody. B, CRE COX-2 binding. Cells were stimulated with 10 μmol/L acrolein, for CREB activation, for 30 minutes and 30 μg samples of whole cell lysates were subjected to Western blotting using anti-phospho-CREB antibody and anti-CREB antibody. B, CRE COX-2 binding. Cells were stimulated with 10 μmol/L acrolein indicated times under serum free conditions. Cells were taken out to prepare nuclear extract, and nuclear proteins were analyzed by EMSA to determine the DNA binding activity of CRE as described under Methods.

Figure 5. CREB activation and binding to COX-2 CRE on acrolein-stimulated HUVECs. A, CREB activation. HUVECs were preincubated with the p38 MAPK inhibitor, SB (SB203580, 10 μmol/L), the MEK inhibitor, PD (PD98059, 40 μmol/L), and the JNK inhibitor, SP (SP600125, 20 μmol/L) for 30 minutes. The cells were then treated 10 μmol/L acrolein, for CREB activation, for 30 minutes and 30 μg samples of whole cell lysates were subjected to Western blotting using anti-phospho-CREB antibody and anti-CREB antibody. B, CRE COX-2 binding. Cells were stimulated with 10 μmol/L acrolein indicated times under serum free conditions. Cells were taken out to prepare nuclear extract, and nuclear proteins were analyzed by EMSA to determine the DNA binding activity of CRE as described under Methods.

Discussion
Cigarette smoking is the leading risk factor in the etiology of atherosclerotic vascular disease.1 Cigarette smoke can damage a number of organ systems; however, ECs are particularly vulnerable. Although it is unclear which smoke constituent is responsible for the deleterious effects, there are over 4000 different chemicals in cigarette smoke, and it is quite likely that a combined insult from several chemical constituents is responsible for the injury.2 In this study, we show that acrolein, a main toxic component of cigarette smoke,2-3 may contribute to the development of vascular disease through COX-2 induction.

The findings presented herein show that acrolein increases COX-2 mRNA, protein, and PG synthesis in HUVECs, time- and dose-dependently. Transient transfections demonstrated that acrolein treatment in HUVECs increases the rate of COX-2 transcription. The increased PG synthesis in HUVECs after treatment with acrolein reflects an increase in functional COX-2 protein, because NS398, a specific inhibitor of COX-2 enzyme activity, effectively blocked PG synthesis in the acrolein-treated cells. To our knowledge, this is the first report that acrolein increases COX-2 expression and PGE2 production in any cell system. The signal transduction cascade that mediates activation of PKCδ, p38 MAPK, and CREB, leading to subsequent COX-2 induction and PGE2 secretion, is diagrammed in supplemental Figure II. Acrolein is present at a level of 238 to 468 μg/cigarette,26 and total aldehyde including acrolein generated by smoking one cigarette, if completely dissolved in the lung lining fluid, could be present at 2 to 3 mmol/L.27 Therefore the concentration of acrolein in this study is considered to be physiological level.

It is well known that COX-2 expression has been linked with activation of MAPK pathways and that the particular signaling pathway involved is dependent on the type of stimuli. In the present study, we demonstrated that acrolein induces COX-2 expression by activating p38 MAPK. In contrast, ERK and JNK did not contribute to acrolein-mediated COX-2 induction. Using siRNA-mediated suppression of p38 MAPK in HUVECs, we were able to gather additional evidence demonstrating role of p38 MAPK in the COX-2 induction by acrolein (Figure 3A).

p38 MAPK plays an important role in the expression of proinflammatory molecules and the regulation of cellular responses during infection and has been widely investigated for an effect on COX-2 at translational and transcriptional levels.28 Interleukin (IL)-1β-induced transcription of COX-2 in a human microvascular endothelial cell line has been shown to require the combinatorial action of transcription factors, such as activated protein-2 (AP2), nuclear factor-IL-6 (NF-IL-6), and cAMP-responsive elements (CRE).29 The CRE element in the COX-2 promoter is necessary for the induction of COX-2 transcription mediated by nitric oxide, proteasome inhibitors, and lipopolysaccharide (LPS).30-32 Our gel shift assay using CRE probe from the COX-2 promoter indicates that CREB binds to a COX-2 CRE (Figure 5B). The involvement of a CRE may be of particular relevance to acrolein-induced COX-2 transcriptional activation.

The activation of PKC induces COX-2 expression in many cell types, such as astrocytic and endothelial cells.33,34 Activation of PKC has been suggested to be a key event in the signal pathway leading to COX-2 expression. In the previous study, we showed that acrolein induce activation of PKCδ in HUVECs.16 In present study we found that COX-2 expression was reduced by a PKC inhibitor, and the PKC activation leads to p38 MAPK activation and COX-2 expression (Figure 4D). Similarly, Kim et al report that epigallocatechin-3-gallate (EGCG)-induced COX-2 expression requires activation of p38 MAPK via PKC pathway in astrocyte and immortalized astrogial cells.33

Acrolein levels are increased in patients with atherosclerosis as well as in cigarette smokers,8,9 which is strongly associated with an increased risk of vascular disease in clinic. Thus increased acrolein levels might be involved in pathogenesis of atherosclerosis. On the other hand, augmented COX-2 expression or PG overproduction in atherosclerotic
lesions has also been reported. Human atheromatous lesions contain COX-2, colocalizing mainly with macrophages of the shoulder region and lipid core periphery. COX-2 expression was also detected in smooth muscle cells and in endothelial cell in atherosclerosis.\textsuperscript{8,9,30,36} Our results show that COX-2 appeared to colocalize with protein-bound acrolein in atherosclerotic blood vessels (Figure 1G and 1H). In addition, very recently, Shao et al reported that acrolein may interfere with atherosclerosis, and with cholesterol transport by modifying specific sites in apoL, resulting in atherogenesis.\textsuperscript{37} Taken together, the possibility that increased levels of acrolein may promote development of atherosclerosis must be seriously considered.

In summary, the present study demonstrates that acrolein, a known toxin in tobacco smoke, stimulates expression of COX-2 and enhances PG synthesis in HUVECs through activation of PKC, p38 MAPK, and CREB pathways. Our finding suggests that acrolein may play an important role in progression of atherosclerosis via an inflammatory response involving COX-2 expression.

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Disclosures
None.

References


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Methods

**Materials** - Acrolein was purchased from the Tokyo Kasei Kogyo Co. (Osaka, Japan). NS-398 and β-actin antibody were obtained from Sigma Co. (St. Louis, USA). The p38 specific inhibitor, SB203580, the ERKs specific inhibitor, PD98059, and the PKC specific inhibitor, rottlerin were purchased from the Calbiochem Co. Inc. (San Diego, CA). The JNK specific inhibitor, SP600125, was obtained from BIOMOL (Plymouth Meeting, PA). Antibodies (Abs) against phospho-specific p38 MAPK, JNK, ERK, PKCδ, CREB and Abs against p38 MAPK, JNK, ERK, PKCδ, and CREB were purchased from Cell Signaling Technology Inc. (Beverly, MA). Anti-COX-1 and COX-2 polyclonal antibodies were purchased from Santa Cruzu Biotechnology (San Diego, CA). Anti-acrolein monoclonal antibody was obtained from Nikken Seil Co. (Osaka, Japan). COX-2 monoclonal antibody and PGE₂ assay kit were purchased from Cayman Co. (Ann Arbor, MI). Transpass R1, R2 and siRNA for p38 MAPK were obtained from New England Biolabs Inc. (Boston, MA). Other chemicals were of the highest grade available.

**Cell Cultures** - Human umbilical vein endothelial cells (HUVEC) were isolated as described previously¹ and were cultured in MCDB131 (Nikken Bio Med Lab Co.) containing 10 % fetal calf serum (FCS), penicillin (100 U/ml), streptomycin sulfate (100 mg/ml), recombinant human fibroblast growth factor (10 ng/ml) and heparin (18 mU/ml) in a humidified atmosphere of 5 % CO₂ at 37 °C. The cells were passaged every 3-4
days. HUVEC were cultured to about 80% confluence and then further incubated with fresh medium containing the above reagents. Throughout these experiments, the cells were used within passages 4-9.

**Animal experiments** - C57/BL6 male mice (8 weeks of age; Charles River, Wilmington, MA) were induced with a single intraperitoneal injection of acrolein (4 mg/kg body weight). Mice were killed by CO₂ inhalation after 24 hr of acrolein injection together with PBS administrated controls. Lung samples were collected and stored at –80°C, before processing for protein analysis.

**Prostaglandin E₂ Production Assay** - PGE₂ in the culture medium was measured with an enzyme immunoassay kit (Cayman Chemical Co. Inc). The assay was performed according to the manufacturer's instructions. Briefly 25 or 50 µl of the medium along with a serial dilution of PGE₂ standard samples were mixed with appropriate amounts of acetylcholinesterase-labeled tracer and PGE₂ antiserum, followed by incubation at room temperature for 18 h. After the wells were emptied, cells were rinsed with wash buffer, 200 µl of Ellman's reagent containing substrate for acetylcholinesterase were added. The enzyme reaction was carried out on a slow shaker at room temperature. The absorbance was read at 415 nm after 1 h incubation.

**siRNA Knockdown of p38 MAPK Expression** - HUVEC were transfected with p38 MAPK siRNA duplexes (NEB), which inhibit expression of p38 MAPK. Forty eighty hours after transfection of 200 nM siRNA using TranspassR2 (NEB), immunoblot analyses were performed. The siRNA was delivered by a lipid-based method supplied from a commercial vendor (NEB) at a final siRNA concentration of 200 nM. After formation of the siRNA-liposome complexes, the mix was added to the HUVEC in
medium for 48 h. Afterward, the medium was aspirated, and complete medium was added back with or without the addition of 10 µM acrolein for a further 16 h.

**Immunofluorescence Staining** - Cells grown in Glass Bottom Microwell Dishes 35 mm culture dishes (MatTek Co.) were incubated for 12 h in presence or absence of acrolein. Cells were washed twice with PBS, fixed with 4% paraformaldehyde in PBS for 20 min at 4 °C, blocked and permeabilized with 1% saponin and 1% bovine serum albumin in PBS for 20 min at 4 °C. The cells were incubated with 1/50-diluted anti COX-2 antibody at room temperature for 2 h with PBS containing 1% bovine serum albumin and 1% saponin. Primary antibody binding was detected with a fluorescein isothiocyanate-labeled goat antibody to rabbit IgG for 4 h at room temperature. Confocal microscopy was done on a LSM410 (Carl Zeiss, Germany) and subsequently handled using Adobe Photoshop.

**Immunohistochemistry** - Atherosclerotic blood vessels were obtained at routine biopsy procedures from patients with atherosclerosis and used for histopathological and immunohistochemical examinations. Tissue samples from each case were fixed in 10% formalin, dehydrated, embedded in paraffin, and stored at room temperature. Serial 4-µm thick sections were cut from paraffin materials and used for hematoxylin-eosin staining or immunohistochemical staining. After deparaffinization and rehydration, the sections were quenched for 15 min with 3% hydrogen peroxide, rinsed in PBS, and boiled for 10 min in 10 mM citrate buffer, pH 6.0, for antigen retrieval. The tissues were pretreated with 5% horse serum in PBS for 30 min at 37 °C and incubated for 60 min at 37 °C with a mouse monoclonal anti-COX-2 antibody (Cayman Co.) at a dilution of 0.1 µg/ml, or the mouse monoclonal anti-acrolein antibody (Nikken Seil Co.) at a dilution of 0.2 µg/ml. Antibody binding was visualized using the SimpleStain kits (Vertor Co.) according to the
manufacturer's instructions. 3,3'-Diaminobenzidine tetrahydrochloride was used as the chromogen, and hematoxylin was used as the counterstain. Sections from which the primary antibodies were omitted served as negative reaction controls. The localizations of acrolein and COX-2 immunoreactivities were verified by consecutive sections stained with hematoxylineosin.

RNA Isolation and Northern Blotting - Total RNA was extracted with acid guanidium thiocyanate-phenol-chloroform as reported previously. Twenty µg of the total RNA were run on a 1 % agarose gel containing 2.2 mol/l formaldehyde. The size-fractionated RNAs were transferred to Zeta-Probe membranes (Bio-Rad Co.) overnight by capillary action. Human COX-2 cDNA (Cayman CO.) was labeled with [α-32P] dCTP (Amersham Pharmacia Biotech.) using random hexanucleotide primers (Multiprime DNA labeling system; Amersham Pharmacia Biotech.). After hybridization with the labeled probes at 42 °C in the presence of 50% formamide, the membrane was washed twice with 2 X sodium chloride-sodium citrate (SSC; 1 X SSC, 15 mM sodium citrate 150 mM NaCl, pH 7.5) which contained 0.1% sodium dodecyl sulfate (SDS) at 50 °C for 30 min, and then washed with 0.2 X SSC with 0.1 % SDS at 50 °C for 10 min. The Kodak X-AR films were exposed for 1-2 days to an intensifying screen at -80 °C. The intensities of the bands on X-ray films were quantitated with a CS-9000 gel scanner (Shimadzu, Japan).

Western Blotting - HUVEC were washed with phosphate-buffer saline (PBS), harvested in lysis buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% (w/v) Triton X-100, 0.5% sodium deoxycholate, 5 mM sodium pyrophosphate, 10 mM NaF, 1 mM Sodium orthovanadate, 10 mM β-glycerophosphate and 1 mM DTT) with protein inhibitor cocktail (Roche Co.). After protein quantification, equal amount of samples (cell
lysates 20-50 µg) were subjected to SDS-PAGE and then transferred to a nitrocellulose membrane (Schleicher & Schuell Co.) under semi-dry conditions using a Transblot apparatus (Bio-Rad Inc.). After blocking by incubation with 5% skim milk in Tris-buffered saline for 1 h at room temperature, the membrane was incubated with the primary antibodies for at 4°C overnight and then washed with Tris-buffered saline containing 0.05% Tween 20. The membrane was further incubated with peroxidase-conjugated goat anti-rabbit IgG (1:3000, Organon Teknika Co.) for 1 h, and then washed, and peroxidase activity was detected by the chemiluminescence method using an ECL kit (Amersham Pharmacia Biotech.).

**Promoter Assay** - HUVEC were seeded at a density of 1 x 10^5 cells/well in 6-well culture dishes and grown for 24 h in medium containing 10% FBS. COX-2 promoter-luciferase plasmid DNA^3^ and pSV-galactosidase were co-transfected into cells using Transpass R1 transfection reagent (NEB) according to the manufacturer's protocol. After 4 h of incubation in serum free, the cells were maintained in 10% FBS/ MCDB131 for another 48 h and then stimulated with acrolein for 6 h. Cells were lysed and luciferase activity was measured in the cellular extracts using an enhanced luciferase assay kit. Luciferase activity was normalized to β-galactosidase activity.

**Gel Shift Assay** - Nuclear extracts were prepared as previously described^4^ . An oligonucleotide whose sequence corresponded to the CRE binding site consensus sequence (sense: 5’-AGAGATTGCCTGACGAGCTAG-3’, antisense: 3’-TCTCTAACGGACTGCCTCTCGATC-5’) was end-labeled with (γ^32^P) ATP and T4 polynucleotide kinase (Promega Co.). Electrophoretic mobility shift assay reaction mixtures contained 20,000 cpm of ^32^P-end-labeled oligonucleotide, 5 µg of nuclear
protein extract, and 1 µg of poly (dL,dC) (Amersham Phamacia Biotech.) in a final volume of 10 µl. Reaction mixtures were resolved on 4% nondenaturing polyacrylamide gel electrophoresis at 200 V for 2 h. Gels were dried and visualized by autoradiography.

Statistical analysis - Data were analyzed by the Student's t-test or ANOVA, and the results were expressed as means ± S.D.

References


**Figure legend for supplemental figures**

**Figure I. Activation of JNK, ERK and p38 MAPK in HUVEC by acrolein**

HUVEC were treated with 10 µM acrolein for the indicated times. Lysates were prepared and 30 µg samples of proteins were analyzed by Western blotting using anti-phospho-JNK (A), ERK (B) or p38MAPK (C) antibodies and anti-JNK, ERK or p38 MAPK antibodies as indicated.

**Figure II. Signaling pathway involved in the induction of COX-2 by acrolein in endothelial cells**

The main signaling pathway for acrolein induction of COX-2 involves activation of PKC, p38 MAPK and CREB.
Supplemental Figure I

Acrolein

0  5  10  30  60 (min)

A

Phospho-JNK

JNK

B

Phospho-ERK

ERK

C

Phospho-p38

p38
Supplemental Figure II

Acrolein

Oxidative stress and/or damaged molecules

PKCδ

p38 MAPK

CREB

CRE

COX-2

PGE₂ production

Inflammatory responses

Atherosclerosis