Identification of a Serum Component That Regulates Cyclooxygenase-2 Gene Expression in Cooperation with 4-Hydroxy-2-nonenal

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Atherosclerosis is a disorder of lipid metabolism as well as a chronic inflammatory disease. Cyclooxygenase-2 (COX-2), an inducible isoform responsible for high levels of prostaglandin production during inflammation and immune responses, mediates a variety of biological actions involved in vascular pathophysiology. We have previously shown that COX-2 gene expression is dramatically induced by a lipid-derived endogenous electrophile, 4-hydroxy-2-nonenal (HNE) (Kumagai, T., Matsion, T., and Uchida, K. (2004) J. Biol. Chem. 279, 48389–48396). In the present study, based on the finding that HNE induced COX-2 expression only in the serum-containing media, we characterized a serum component essential for the HNE-induced COX-2 induction and found that low density lipoprotein (LDL) that had been denatured by freeze-thawing or oxidized LDL might be involved in the COX-2 induction. Moreover, we characterized the cellular events triggered by the combined stimulus of HNE and oxidized LDL and established that COX-2 induction is regulated by two sets of signaling mechanisms, one for the up-regulation of the scavenger receptor CD36 by HNE and one for the CD36-mediated COX induction by oxidized LDL. These findings represent a demonstration of a link between lipoprotein modification and activation of the inflammatory potential of macrophages.

Atherosclerosis is a disorder of lipid metabolism as well as a chronic inflammatory disease. Monocyte-derived macrophages play a prominent role in the formation and progression of atherosclerotic plaque, particularly after their transformation into foam cells. When activated by inflammatory stimuli, the macrophages synthesize and secrete various mediators, including cytokines, prothrombotic substances, and eicosanoids, which cause the clinical manifestations and acute clinical complications of atherosclerosis. The eicosanoids derived from the metabolism of arachidonate have been extensively investigated because several studies have focused on their close relation to atherogenesis (1, 2). In macrophages as well as in other cell types arachidonate metabolites are synthesized by the cyclooxygenase enzyme. Presently, two isoforms of cyclooxygenase have been identified: cyclooxygenase-1 (COX-1), which is the constitutive form, and cyclooxygenase-2 (COX-2), which is the inducible form. COX-1 is present under normal conditions in most tissues and is responsible for housekeeping functions. On the other hand, COX-2 is not normally present under basal conditions or is present in very low amounts. COX-2 is rapidly induced by various stimuli, including proinflammatory cytokines, such as interleukin-1β and tumor necrosis factor-α, growth factors, and tumor promoters, to result in prostaglandin synthesis associated with inflammation and carcinogenesis (3). Substantial evidence indicates that unregulated COX-2 expression and prostaglandin synthesis influence chronic inflammatory conditions, including atherosclerosis and its complications (3–5).

Various lines of evidence indicate that an important part of the pathogenesis of atherosclerosis is the modification of plasma low density lipoproteins (LDL) (6, 7). A large number of pro-inflammatory and pro-atherogenic properties has been ascribed to oxidatively modified LDL (oxLDL) and their components (8). In particular, there is considerable evidence to support the role of oxidized fatty acids originating from the oxLDL as important signaling molecules in the context of the atherosclerotic lesion. Podrez et al. (9) have recently shown that oxLDL components, such as oxidized phosphatidylcholines, serve as endogenous ligands for the scavenger receptor, CD36, facilitating macrophage cholesterol accumulation and foam

The abbreviations used are: COX-2, cyclooxygenase-2; EGFR, epidermal growth factor receptor; FBS, fetal bovine serum; HNE, 4-hydroxy-2-nonenal; LDL, low density lipoproteins; LPS, lipopolysaccharide; oxLDL, oxidized low density lipoprotein; p38 MAPK, p38 mitogen-activated protein kinase; CHO, Chinese hamster ovary; RT, reverse transcription; siRNA, small interfering RNA.
cell formation. Nagy et al. (10) have shown that the oxidized fatty acids, such as 9- and 13-hydroxyoctadecadienoic acids and 15-hydroxyeicosatetraenoic acid, regulate the macrophage gene expression through the ligand activation of the peroxisome proliferators-activated receptor γ. In addition, the lipid peroxidation-derived short-chain aldehydes, such as acrolein and 4-hydroxy-2-nonenal (HNE) (Fig. 1), have been shown to modulate the NF-κB-dependent signaling pathways, which play an important role in gene regulation during inflammatory and immune responses (11). The oxidized lipids generated during oxidative modification of LDL are, therefore, likely to be involved in the process of macrophage transformation into the foam cells during atherogenesis.

In view of the observation that the increased eicosanoid production is closely associated with atherogenesis (1, 2), we hypothesized that an oxidized LDL component might be involved in the up-regulation of the prostanoid biosynthesis. In our previous studies (12), we demonstrated that COX-2 colocalizes with protein-bound HNE in macrophage-derived foam cells within atheromatous lesions. Moreover, we evaluated the effect of the oxidized fatty acid metabolites on COX-2 induction in RAW264.7 macrophages and identified HNE as the most potent inducer of COX-2 expression. These findings represented a demonstration of a link between the oxidized fatty acid metabolites and the activation of COX-2 gene expression in macrophages. In the present study we further extend our study on the molecular mechanism of the COX-2 induction by HNE and unequivocally show that a serum component is essential for COX-2 induction. In addition, signal transduction mechanisms involved in the COX-2 expression stimulated by the combination of HNE and the serum component are shown.

**EXPERIMENTAL PROCEDURES**

**Materials**—4-Hydroxy-2-nonenal (HNE) and the COX-2-specific inhibitor, NS398, were purchased from the Cayman Chemical Co. (Ann Arbor, MI). 4-Oxo-2-nonenal was synthesized by the oxidation of HNE dimethyl acetal with pyridinium dichlorochromate followed by HCl hydrolysis (13). Acrolein was purchased from Sigma. The p38 mitogen-activated protein kinase (MAPK)-specific inhibitor, SB203580, was from BIOMOL (Plymouth Meeting, PA). The Src-tyrosine kinase inhibitor, PP2, was purchased from Calbiochem. Goat anti-COX-2 polyclonal antibodies M19 specific for human, rat, and mouse COX-2 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Phycocerythrin-conjugated anti-mouse CD36 monoclonal antibodies were from BioLegend (San Diego, CA). Anti-rabbit IgG immunoglobulin-conjugated horseradish peroxidase, enhanced chemiluminescence (ECL) Western blotting detection reagents, and Hybond ECL nitrocellulose membranes were obtained from Amersham Biosciences. Protein concentration was measured using the BCA protein assay reagent obtained from Pierce. Centripus centrifugal filters with a molecular weight cutoff of 30,000 were purchased from Millipore (Bedford, MA).

**Preparation of Human Serum**—Blood from healthy volunteers was allowed to clot for 60 min at room temperature and then placed overnight at 4 °C. Serum was obtained by centrifugation at 40,000 × g for 10 min at 4 °C and stored at 4 °C until use.

**Isolation of Lipoproteins and Preparation of Modified LDL**—Chylomicron and very low density lipoprotein (VLDL) (ρ<1.006 g/ml), LDL (1.006 < ρ < 1.063 g/ml), high density lipoprotein (HDL) (1.063 < ρ < 1.21), and lipoprotein-deficient serum (Lp-D) (ρ > 1.21) were isolated from fetal bovine serum (FBS) or normal human serum by sequential ultracentrifugation. They were extensively dialyzed against phosphate-buffered saline (PBS) containing 0.3 mM EDTA, sterilized by filtration through a 0.22-μm filter, and stored under argon at 4 °C. Oxidation of LDL (1 mg of protein/ml) was initiated by the addition of CuSO4 (20 μM) followed by incubation continued for up to 24 h at 37 °C under sterile conditions. Reactions were terminated by addition of EDTA (0.2 mM). Acetyl-LDL was prepared according to the method of Basu et al. (14). Freeze-thawed LDL was prepared by incubating LDL in PBS containing 0.3 mM EDTA overnight at −20 °C followed by thawing at room temperature. LDL that had been stored at 4 °C was used as control.

**Centrifugal Serum Ultrafiltration**—The centrifugal filter membranes were rinsed and used according to the manufacturer’s specifications. Ten microliters of human serum was diluted by the addition of 50 ml of 25 mM NH4HCO3, pH 8.2, 20% (v/v) acetonitrile, and applied onto a Centrisil centrifugal concentrator membrane (molecular weight cutoff of 30,000).

**Cell Culture**—The murine macrophage cell line RAW264.7 was kindly given by Dr. Murakami (Kyoto University, Kyoto, Japan). The cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% FBS, penicillin (100 units/ml), streptomycin (100 μg/ml), l-glutamine (588 μg/ml), and 0.16% NaHCO3. The cells were incubated under humidified atmosphere of 95% O2 and 5% CO2 at 37 °C. The CD36-overexpressing cells (CHO-CD36 cells) were prepared by transfecting the human CD36 cDNA into Chinese hamster ovary (CHO)-K1 cells as previously described (15).

**Agarose Gel Electrophoresis/Immunoblot Analysis**—Agarose gel electrophoresis of LDL was performed with the Helena TITAN GEL high resolution protein system (Helena Laboratories, Saitama, Japan). LDL was visualized by Fast Red 7B staining.

**Prostaglandin E2 Assay**—A solid phase enzyme immunoassay (Cayman) was performed as suggested by the manufacturer, and the prostaglandin E2 level was determined using a standard curve and a linear log-logit transformation.

**Immunoblot Analysis**—Cells were washed twice with phosphate-buffered saline, pH 7.0, and lysed with lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 100 μg/ml phenylmethylsulfonyl fluoride). After protein quantification, equal amounts of protein (total protein, 20–50 μg) were boiled with Laemmli sample buffer for 5 min at 100 °C (16). The samples were run on 10% SDS-polyacrylamide gels, transferred to a nitrocellulose membrane, incubated with 5% skim milk in TTBS (Tris-buffered saline containing 10% Tween 20) for blocking, washed, and treated with the primary antibodies. After washing with TTBS, the blots were further incubated for 1 h at room temperature with IgG antibody coupled to horseradish peroxidase in TTBS. Blots were then washed 3 times in TTBS before visualization. An ECL kit was used for detection.

**RNA Isolation and Reverse Transcription (RT)-PCR**—Total RNA was isolated with TRIzol reagent (Invitrogen). The RNA
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concentration was determined by measuring the absorbance at 260 nm. The RT reaction was performed with 5 μg of total RNA and an oligo(dT) primer using the First-strand cDNA synthesis kit. PCR reactions were carried out using 0.5 μl of cDNA in 25 μl of 10 mm Tris-HCl, pH 8.3, containing 50 mg KCl, 0.1% Triton X-100, 200 μm dNTPs, 1 μm concentrations of each forward and reverse primer, and 2 units of TaqDNA polymerase (Toyobo Co., Osaka, Japan). The following primers were used: COX-2, (forward (F)) 5′-CCCCATGCTGAGCAAAGCC-3′ and (reverse (R)) 5′-CTAGGGGTATTATGCTCATGAC-3′; glyceraldehyde-3-phosphate dehydrogenase, (F) 5′-AACCCATCCACATCTTCCAGGAGC-3′ and (R) 5′-CAGCTCTCTGTGAAGCAGTATG-3′; LDL receptor, (F) 5′-GAAATCCATGTCGAGATGGCTCT-3′ and (R) 5′-CTTCAGTCTTCAACAGAGCTGGGTC-3′; CD36, (F) 5′-CCCACTTTGGTGGAAAAGC-3′ and (R) 5′-GAACCTATTGTGTAGTGGCAGTGAT-3′; SR-A1, (F) 5′-CTCCACGAGGTCACACAGAGCTGG-3′ and (R) 5′-ATGCACTTGTGTTTTGAA-3′; SR-B1, (F) 5′-CTCCAGGATCATGGCCTTCCATA-3′ and (R) 5′-CTGGTTCTTCAACAGGACCTGCA-3′; COX-2, (F) 5′-GAATTCGAAGATGCAATGGCTCCT-3′ and (R) 5′-CTCCCAGACATGCTCCTTCCATT-3′; macroisolation, (F) 5′-CTAGGCTTCTGACATCCTTCT-3′ and (R) 5′-TTCCACCCGCACTTGTAGTCC-3′.

Flow Cytometric Analysis of CD36 Expression—RAW264.7 cells were treated as indicated, and CD36 expression was analyzed by fluorescence-assisted cell sorting (Coulter Epics XL flow cytometer). CD36 expression was measured using phycoerythrin-conjugated anti-mouse CD36 monoclonal antibody diluted 1:50 in phosphate-buffered saline and 0.5% bovine serum albumin. Appropriate isotype matched control fluorescent antibodies were also used to measure nonspecific binding. Approximately 10,000 cells were analyzed in each sample.

RNA Interference Using siRNA—The Stealth siRNA oligonucleotide was synthesized by Invitrogen with the following sequence complementary to mouse CD36 mRNA (GenBankTM accession no. NM007643); the oligonucleotides of mouse CD36 siRNA were: sense, 5′-UAGCUUUGGCAAUUGACC-3′; antisense, 5′-GGAAUUUGCUACUUGGCCGCA-3′. The Stealth siRNA negative control duplex (Invitrogen) was used as a control oligonucleotide. RAW264.7 cells were seeded at 50–60% density the day before transfection. Cells were transfected with Lipofectamine 2000™; 4 μl of siRNA stock (20 μM) and 8 μl of Lipofectamine 2000™ were each diluted with 250 μl of Opti-MEM I. After 5 min at room temperature, they were combined and incubated for 20 min. The reaction mixtures were overlaid on the cell culture for 24 h. The medium was then changed to fresh medium containing 10% FBS.

Lipid Binding Assay—RAW264.7 cells were incubated in the dark with oxLDL labeled with 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine at a final concentration of 50 μg/ml. LDL protein for 2 h at 4 °C on a rotary shaker. The cells were washed twice, resuspended in PBS, and then directly analyzed by flow cytometry. Final cell concentration was 1 × 10⁶ cells/ml. Each experiment was performed in duplicate.

Immunohistochemistry—Aortic wall samples were obtained at autopsy from five cases of arterial atherosclerosis without diabetes mellitus or any other arterial disorders, performed after their family members granted informed consent. Tissue samples of each case were processed for making frozen materials and used for hematoxylin-eosin stain and immunohistochemical stain. The samples were embedded in OCT™ compound (Sakura Fine Technical Co., Tokyo, Japan), stored at −80 °C, and cut into 6-μm-thick sections by a cryostat. The sections were rehydrated in distilled water, quenched with 3% hydrogen peroxide for 15 min at 4 °C, rinsed in PBS, and pre-treated with 3% nonimmune serum followed by blocking endogenous avidin/biotin activity using a kit (Vector Laboratories, Burlingame, CA) according to the manufacturer’s instructions. The sections were then incubated overnight at 4 °C with a mouse monoclonal anti-CD68 antibody, rabbit polyclonal anti-CD36 antibody (Santa Cruz, CA), or the mouse monoclonal anti-protein-bound HNE antibody HNE2 (17). Immunoreaction was visualized by the avidin-biotin-immunoperoxidase complex method using the appropriate Vectastain ABC kit (Vector). Immunostained sections were counterstained with hematoxylin. Sections from which the primary antibodies were omitted served as negative reaction controls. The localization of protein-bound HNE and CD36 immunoreactivities was verified by consecutive sections stained with hematoxylin and eosin and immunostained for CD68.

Double immunofluorescence was performed to determine whether protein-bound HNE and CD36 immunoreactivities are localized in foamy macrophages. In brief, sections were simultaneously incubated with primary antibodies against protein-bound HNE and CD36 followed by secondary antibodies such as fluorescein isothiocyanate-conjugated donkey anti-mouse IgG and Cy3-conjugated donkey anti-rabbit IgG each at a dilution of 1:200 (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). Double-stained specimens were examined with a fluorescence microscope (Axiovert S100, Carl Zeiss, Jena, Germany) or a confocal laser microscope (TCS-SL; Leica, Heidelberg, Germany).

RESULTS

A Serum Protein Is Essential for Induction of COX-2 by HNE—In the signaling pathway for lipopolysaccharide (LPS)-induced COX-2 induction in macrophages, LPS first binds to LPS-binding protein, a secreted serum protein that transfers LPS to an important LPS binding receptor, CD14, thereby enhancing the immune response to LPS (18). This finding led us to examine the involvement of serum in the HNE-induced COX-2 expression. As shown in Fig. 1B, when RAW264.7 macrophages were treated with HNE (0–75 μM) for 4 h in the presence and absence of the serum (10% FBS), COX-2 induction was observed only in the serum-containing media. In addition, the effect of HNE on the COX-2 induction was enhanced by the increase in the serum concentration (Fig. 1C). A human serum that had been commercially obtained showed a similar effect (data not shown). Heat treatment of the serum at boiling temperature for 5 min resulted in the complete loss of the COX-2 inducing activity (Fig. 1D), suggesting that the active component in the serum is protein nature. To evaluate the molecular weight of the active component in the serum essential for COX-2 induction by HNE, we fractionated the pooled commercial human serum with polycarbonate membrane-based centrifugal ultrafiltration (Centricon), and each fraction
than those of the albumin fractions (supplementary Fig. S1). The specific activities of the lipoprotein fractions were much greater than those of the albumin fractions. The ratio of the COX-2 inducibility to the albumin fraction (fractions 13–19) and the albumin fraction (fractions 24–29). The ratio of the COX-2 inducibility to the lipoprotein fraction (fractions 13–19) and the albumin fraction was detected mainly in two fractions corresponding to the protein concentrations of each fraction indicated that the albumin fraction showed the strongest COX-2 inducibility (Fig. 2D). These findings agree with the observation that the active component is a relatively large protein with a molecular weight of >100 (Fig. 1E). Thus, LDL was tentatively identified as the serum components essential for the induction of COX-2 by HNE.

**Identification of a Bona Fide Active Component Essential for COX-2 Induction**—To further confirm the COX-2 inducibility of LDL, we freshly prepared the serum from healthy volunteers and examined its COX-2-inducing activity in the presence of HNE. However, although the bovine and human sera that had been commercially obtained significantly induced COX-2 gene expression in the presence of HNE (Fig. 3A, lanes 2 and 3), the freshly prepared human serum had no effect (Fig. 3A, lane 4). The data led us to the assumption that the LDL in the pooled commercial sera might acquire the COX-2 inducibility through denaturation. Indeed, the agarose gel electrophoresis demonstrated that the LDL prepared from the pooled commercial serum was more electronegative than the freshly prepared LDL (Fig. 3B). It is, therefore, likely that the LDL from different batches contains different levels of denatured LDL, which along with other properties may contribute to the extent of COX-2 induction. In addition, the LDL that had been freeze-thawed significantly induced COX-2 in the presence of HNE (Fig. 3C). COX-2 induction by HNE in the serum-supplemented medium was also reproduced by the treatment of the cells with denatured LDL, such as acetylated LDL, in the presence of HNE (Fig. 3D). In the absence of HNE, however, modified LDL, including freeze-thawed LDL, acetyl-LDL, and covalently modified LDL with HNE or its analog 4-oxo-2-nonenal, were inactive on COX-2 induction. These results indicate that denatured LDL might represent a bona fide serum component essential for the induction of COX-2 by HNE.

**Separation of Serum Components Essential for Induction of COX-2**—We then attempted to identify the active component contained in FBS. To this end we separated and fractionated the serum by gel filtration chromatography on a Hi Prep 16/60 Sephacryl S-300 column, and aliquots of each fraction were examined for COX-2 inducibility in the presence and absence of HNE. As shown in Fig. 2 (panels A and B), the COX-2-inducing activity was detected mainly in two fractions corresponding to the lipoprotein fraction (fractions 13–19) and the albumin fraction (fractions 24–29). The ratio of the COX-2 inducibility to the protein concentrations of each fraction indicated that specific activities of the lipoprotein fractions were much greater than those of the albumin fractions (supplementary Fig. S1). When the lipoprotein fractions were collected and subjected to the COX-2-inducing activity in the presence and absence of HNE, they induced COX-2 up-regulation in a dose-dependent manner (Fig. 2C). To identify which classes of lipoproteins are essential for the COX-2 induction by HNE, we fractionated the serum into very low density lipoprotein, LDL, high density lipoprotein, and other (lipoprotein-free) fractions by sequential ultracentrifugation and found that the LDL fractions showed the strongest COX-2 inducibility (Fig. 2D). These findings agree with the observation that the active component is a relatively large protein with a molecular weight of >100 (Fig. 1E). Thus, LDL was tentatively identified as the serum components essential for the induction of COX-2 by HNE.

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**FIGURE 1. Serum-dependent induction of COX-2 by HNE in RAW264.7 macrophages.** A, chemical structure of HNE. B, dose-dependent induction of COX-2 by HNE (0–75 μM) in the presence and absence of serum. C, dose-dependent induction of COX-2 by serum (0–20%) in the presence and absence of 50 μM HNE. D, dose-dependent induction of COX-2 by heated and non-heated serum (0–20%) in the presence of 50 μM HNE. E, COX-2 inducibility of the serum protein fractions prepared by centrifugal ultrafiltration. The commercial human serum was fractionated with polycarbonate membrane-based centrifugal ultrafiltration (Centricon), and each fraction was examined for COX-2 inducibility in the presence and absence of 50 μM HNE. In panels B–E, cells were incubated for 4 h, and the protein expression levels were determined by immunoblot analysis.

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of COX-2 was also observed in primary murine peritoneal macrophages exposed to these two molecules (supplemental Fig. S2). Moreover, the observation that the incubation of oxLDL with HNE before the exposure to RAW264.7 macrophages resulted in a significant decrease in the COX-2 inducibility (supplementary Fig. S3) suggested that two molecules, HNE and oxLDL, might act independently on the cells and cooperatively activate cell signaling mechanisms for COX-2 gene expression.

Involvement of the Scavenger Receptor CD36—The appearance of lipid-laden foam cells is one of the hallmarks of fatty streaks and atherosclerotic plaques. The transformation of macrophages into lipid-laden foam cells is most likely the result of receptor-mediated uptake of cholesterol-rich particles. The identification of denatured LDL, including oxLDL, as an active component essential for the induction of COX-2 by HNE led us to the assumption that scavenger receptor(s) might be involved in the signaling mechanism underlying the COX-2 expression. Hence, we treated RAW264.7 macrophages with HNE for 1 h in the presence and absence of oxLDL, and the mRNA level of each scavenger receptor was determined by RT-PCR. Although stimulation with the combination of HNE and oxLDL resulted in a marked induction of both COX-2 and CD36 mRNAs (Fig. 4B), CD36 expression was significantly induced by treatment with HNE alone (Fig. 4A). No significant induction of CD36 was observed when cells were treated with oxLDL alone (data not shown). To investigate whether the enhanced CD36 gene expression is associated with the expression of functionally active CD36 protein, we examined cell surface expression of CD36 and binding of oxLDL to the cells treated with or without HNE by flow cytometry. As shown in Fig. 4C, the amount of immunoreactive CD36 on the cells was significantly increased after treatment with HNE. In addition, a strong induction of surface CD36 expression was associated with the increased binding of oxLDL to the cells as examined by flow cytometry using an anti-apoB monoclonal antibody (Fig. 4D).

Furthermore, the pathohistologic location of the protein-bound HNE and CD36 in human atherosclerotic lesions was examined. The non-atherosclerotic arterial wall had little or no CD36 and protein-bound HNE, whereas in and around advanced atherosclerotic core region, protein-bound HNE and CD36 localized in the cytoplasm of foamy macrophages, which were identified by histopathological and immunohistochemical features on CD68-immunostained sections (Fig. 5A). No immunoreaction product deposits were detected in sections with omission of the primary antibodies (data not shown). Results from double label experiments also provided evidence for the colocalization of protein-bound HNE with CD36 (Fig. 5B). These data and the previous observations that under oxidative stress conditions HNE can accumulate at even higher concentrations of 10 μM to 5 mM both in vivo and in vitro (11, 21) suggest that the amounts of free HNE generated in vivo, atherosclerotic lesions in particular, may be sufficient for it to play a role in stimulating most of the responses, including CD36 gene expression, in macrophages.

Functional Association of the HNE-induced CD36 Up-regulation with COX-2 Induction—We determined if CD36 is functionally associated with the induction of COX-2. As shown in Fig. 6A, the reduction of CD36 expression by treatment with CD36 siRNA resulted in the reduced expression of COX-2 in RAW264.7 macrophages exposed to HNE and oxLDL. In addition, using CD36-overexpressed CHO-CD36 cells, we examined the effect of COX-2 overexpression upon induction of COX-2 by oxLDL in the presence and absence of HNE. As shown in Fig. 6B, COX-2 was induced only by the combined stimulus of HNE and oxLDL in wild-type CHO-K1 cells, whereas stable overexpression of CD36 resulted in the induc-
although the mechanism of COX-2 transcription in macrophage cells is complex, resolving the complicated network of regulatory events controlling COX-2 expression during inflammation will likely uncover new potential clinical targets for lowering COX-2 activity in a cell-specific manner. In the present study we found that the serum was essential for the COX-2 expression induced by a major lipid peroxidation product. Separation of the human serum by gel filtration tentatively identified lipoproteins such as very low density lipoprotein and LDL as the co-inducers of COX-2 expression. Of interest, however, LDL derived from pooled serum but not LDL freshly prepared from human serum; LDL derived from serum of healthy subjects induced COX-2 in the presence of HNE. In addition, several modified forms of electrophoretically identified lipoproteins such as very low density lipoprotein and LDL. The oxidation of LDL was performed by incubating LDL (1 mg/ml) with CuSO4 (5 μM) in 50 mM sodium phosphate buffer, pH 7.4, at 37 °C. F. COX-2-inducibility of oxLDL in the presence and absence of HNE. RAW264.7 macrophages were treated with native or oxLDL (100 μg/ml) for 4 h in the presence and absence of 50 μM HNE. G. Enhanced production of prostaglandin E2 (PGE2). RAW264.7 macrophages were treated with oxLDL (100 μg/ml) and HNE (50 μM) in the presence and absence of 10 μM NS398 for 24 h. 

DISCUSSION

A wide variety of extracellular molecules, including LPS, cytokines, and growth factors, increase the expression of COX-2. The expression of COX-2 is complex, resolving the complicated network of regulatory events controlling COX-2 expression during inflammation will likely uncover new potential clinical targets for lowering COX-2 activity in a cell-specific manner. In the present study we found that the serum was essential for the COX-2 expression induced by a major lipid peroxidation product. Separation of the human serum by gel filtration tentatively identified lipoproteins such as very low density lipoprotein and LDL as the co-inducers of COX-2 expression. Of interest, however, LDL derived from pooled serum but not LDL freshly prepared from human serum induced COX-2 in the presence of HNE. In addition, several modified forms of electrophoretically identified lipoproteins such as very low density lipoprotein and LDL as the co-inducers of COX-2 expression. Of interest, however, LDL derived from pooled serum but not LDL freshly prepared from human serum induced COX-2 in the presence of HNE. In addition, several modified forms of electrophoretically identified lipoproteins such as very low density lipoprotein and LDL as the co-inducers of COX-2 expression. Of interest, however, LDL derived from pooled serum but not LDL freshly prepared from human serum induced COX-2 in the presence of HNE. In addition, several modified forms of electrophoretically identified lipoproteins such as very low density lipoprotein and LDL as the co-inducers of COX-2 expression.
denatured LDL might represent a bona fide active component essential for the induction of COX-2 by HNE. It has been shown that human plasma contains a denatured, electronegative LDL subfraction that possesses atherogenic properties and is associated with increased cardiovascular disease risk (23, 24). Most mechanisms that describe the formation of such modified LDLs involve the oxidative modification of particles (19, 20). The oxidized LDL has been suggested to play a causative role in atherosclerotic plaque formation (6, 25). Although the detailed mechanism for the oxidative modification of lipoproteins has not yet been established, it is generally accepted that the primary generation of lipid hydroperoxides initiates a reaction cascade leading to the rapid propagation and amplification of the number of reactive oxygen species formed; this ultimately leads to extensive fragmentation of the fatty acid chains and conversion of the LDL into a more atherogenic form (26). A large number of pro-inflammatory and pro-atherogenic properties has been ascribed to oxLDL and its components (8).

The identification of denatured LDL as the inducer of COX-2 led us to the assumption that scavenger receptor(s) might be involved in the induction mechanisms. Hence, we examined the changes in the mRNA levels of scavenger receptors in RAW264.7 macrophages exposed to HNE in the presence and absence of oxLDL and found that exposure of macrophages to HNE alone resulted in the increased expression of CD36, a major receptor responsible for the binding and uptake of modified LDL in macrophages (Fig. 4). To further obtain evidence for the involvement of HNE in the CD36 expression in vivo, we examined the pathohistologic location of the protein-bound HNE and CD36 in human atherosclerotic lesions and observed the colocalization of protein-bound HNE and CD36 immunoreactivities in foamy macrophages (Fig. 5). These data led us to the speculation that the HNE-induced CD36 expression might be associated with the induction of COX-2 expression. This hypothesis was supported at least in part by our observations (Fig. 6) that (i) the reduction of CD36 expression by treatment with CD36 siRNA resulted in the reduced expression of COX-2 induced by the combination of HNE and oxLDL and (ii) overexpression of CD36 resulted in the enhanced expression of COX-2 induced by oxLDL alone. Our findings suggest the existence of a mechanism whereby the oxidized lipid promotes the uptake of denatured LDL by increasing the level of the scavenger receptor expression, leading to the expression of COX-2. This mechanism may explain at least some of the biological effects of oxLDL. These observations strongly suggested that
the oxidized lipid component of oxLDL particles could function as an endogenous inducer of CD36 gene expression. This association of HNE with CD36 may be consistent with the previously defined role for oxLDL in the regulation of CD36 expression (10).

CD36, a glycosylated surface receptor, belongs to a class B scavenger receptor and is localized in lipid rafts of the plasma membrane and in mitochondria (27–29). It is expressed on macrophages, microglia, microvascular endothelium, cardiac and skeletal muscle, adipocytes, and platelets and recognizes a variety of ligands, including oxLDL, advanced glycation end products, long chain fatty acids, fibrillar β-amyloid, thrombospondins, and cells undergoing apoptosis (30–32). This multifunctional receptor plays a pivotal role in angiogenesis, inflammation, apoptosis, and lipid metabolism (30, 32). In addition, it also plays important physiological roles in the development of atherosclerosis and a number of human malignancies. CD36 expression on macrophage CD36 signaling may vary between different cellular contexts.

It is challenging to define a target molecule that triggers signal transduction pathways leading to CD36 expression by HNE. Based on our previous observations that the inhibitors of the EGFR-tyrosine kinases significantly suppressed the HNE-induced COX-2 expression (12), we speculate that EGFR may represent one of the upstream targets of HNE in CD36 gene expression. The EGFR, a transmembrane receptor-tyrosine kinase shared by several growth factors, is implicated in various biological processes such as cell proliferation or differentiation and has been suggested to be involved in the genesis or progression of atherosclerosis and a number of human malignancies. EGFR activation is associated with the stimulation of its intrinsic tyrosine kinase, with autophosphorylation of its own tyrosine residues and with phosphorylation of intracellular substrate proteins. In our preliminary experiments we have observed that HNE up-regulated the catalytic actions of EGFR for autophosphorylation in RAW264.7 macrophages and that the inhibitors of EGFR-tyrosine kinase down-regulated the HNE-induced CD36 gene expression.3 In addition, both acrolein and 4-oxo-2-nonenal, possessing similar electrophilic properties as HNE, were inactive on CD36 expression (supplementary Fig. S5). Although the reason for these apparently contradictory results remains unclear, one possibility may be that the induction of CD36 by HNE requires integration of multiple signaling pathways, and therefore, the net effect of NF-E2-related factor-2 depletion and Kelch-like ECH-associated protein 1 overexpression on macrophage CD36 signaling may vary between different cellular contexts.

In conclusion, we showed that serum was essential for the oxidation of the scavenger receptor CD36. The up-regulation of CD36 is accompanied by the enhanced uptake of denatured LDL through CD36 and promotes the CD36-mediated COX-2 induction by denatured LDL.

FIGURE 7. A proposed mechanism for induction of COX-2 by the combined stimulus of HNE and denatured LDL. HNE, generated during oxidative stress and LDL oxidation, up-regulates gene expression of the scavenger receptor CD36. The up-regulation of CD36 is accompanied by the enhanced uptake of denatured LDL through CD36 and promotes the CD36-mediated COX-2 induction by denatured LDL.

It has been shown that CD36 associates with a signaling complex containing Src-tyrosine kinases and with activation of the kinases and subsequent downstream activation of p38 MAPK (22). We, therefore, hypothesized that binding of oxLDL to CD36 followed by activation of these protein kinases results in the induction of COX-2 up-regulation. Indeed, our experiments demonstrated that the pharmacological inhibitors of Src-tyrosine kinases and p38 MAPK suppressed the HNE/oxLDL-induced COX-2 expression in a dose-dependent manner (supplementary Fig. S4). In addition, the Src-tyrosine kinase inhibitor completely suppressed the phosphorylation of p38 MAPK by HNE/oxLDL (supplementary Fig. S4). These data suggest that the exposure of RAW264.7 macrophages to oxLDL leads to recruitment of a Src-tyrosine kinase to a CD36 membrane complex with activation of the kinase and subsequent downstream activation of p38 MAPK leading to the induction of COX-2 gene expression.

In conclusion, we showed that serum was essential for the HNE-induced COX-2 gene expression in RAW264.7 macrophages and identified denatured LDL, including oxLDL, as

3 M. Kanayama and K. Uchida, unpublished data.
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active events. In addition, we characterized the cellular events triggered by the combination of HNE and oxLDL and established that they cooperatively induced COX-2 gene expression through a novel mechanism, by which HNE up-regulates gene expression of the scavenger receptor CD36 and promotes the CD36-mediated COX-2 induction by denatured LDL (Fig. 7). These findings represent a further demonstration of a link between the oxidative modification of LDL and the activation of the inflammatory potential of macrophages. The observed effect could be relevant in atheromata, where close contact between macrophages and oxidized lipoproteins, containing a large amount of oxidized lipid components, might ultimately result in the development of an inflammatory response. This phenomenon may, thus, represent an important contributing feature in an early step in the process of macrophage transformation into the foam cells composing the fatty streak, a primary histologic aspect of incipient atherosclerosis.

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