Mechanisms of low-density lipoprotein-induced expression of connective tissue growth factor in human aortic endothelial cells

Mimi Sohn, Yan Tan, Bing Wang, Richard L. Klein, Maria Trojanowska, and Ayad A. Jaffa

Origins of 1Endocrinology, Diabetes, and Medical Genetics and of 2Rheumatology and Immunology, Department of Medicine, Medical University of South Carolina, and 3The Ralph H. Johnson Veterans Affairs Medical Center, Charleston, South Carolina

Submitted 7 December 2004; accepted in final form 1 November 2005

Hyperlipidemia is associated with increased morbidity and mortality derived mainly from cardiovascular complications. Indirect evidence supporting this notion is provided by the fact that high levels of expression of CTGF mRNA and protein concentrations occur in VSMC and endothelial cells of advanced human atherosclerotic lesions (31). In addition, VSMC expressing CTGF were localized predominantly in areas with extracellular matrix production and especially in areas around the fibrous cap, thus indicating that CTGF may regulate the production of matrix proteins in these cells (31).

CTGF was originally identified as a product of human umbilical vein endothelial cells that were both chemotactic and mitogenic for fibroblasts (5). It is now known that CTGF belongs to a new gene family, CCN (named after prototype members of this family, CTGF, cyr61, and nov) (4). The molecular weight of CTGF-like factors varies between 35 and 40 kDa, and the structure of these molecules consists of four modules: an NH2-terminal insulin-like growth factor (IGF) binding protein-like domain, a von Willebrand factor type C repeat domain, a thrombospondin type 1 repeat domain, and a COOH-terminal dimerization domain (4). An emerging role of CTGF is that of a prosclerotic factor. CTGF was shown to be expressed in atherosclerotic lesions and intima vascular smooth muscle cell (VSMC) proliferation, migration, and extracellular matrix (ECM) deposition in the vessel wall (9, 10, 17, 34). In addition, endothelial cells tend to lose their physiological role as a semipermeable membrane barrier that separates the blood cells and soluble components, such as proteins or lipoprotein complexes, from the cells of the underlying the endothelium. This stage in the development of atherosclerosis is a complex process involving interactions between lipids and activated cells of the immune system, T cells, monocytes, or macrophages, which then convert into foam cells (40).

Although the association of hyperlipidemia and atherosclerosis is well established, the cellular signaling mechanisms that promote atherosclerosis are still undefined. In this regard, the cytokine transforming growth factor-β (TGF-β) has been shown to play a pivotal role in vessel wall remodeling after vascular injury (41). Recent studies have implicated a key role for the cytokine TGF-β as a mediator of vessel wall remodeling after endothelial injury (3, 28). Recent evidence indicates that TGF-β may mediate its fibrotic effects via activation of CTGF (13). The expression of CTGF is induced by TGF-β in VSMC, indicating that CTGF may act along a causal pathway for atherosclerosis. Indirect evidence supporting this notion is provided by the fact that high levels of expression of CTGF mRNA and protein concentrations occur in VSMC and endothelial cells of advanced human atherosclerotic lesions (31). In addition, VSMC expressing CTGF were localized predominantly in areas with extracellular matrix production and especially in areas around the fibrous cap, thus indicating that CTGF may regulate the production of matrix proteins in these cells (31).

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(35) and to mediate adherence and migration of monocytes, VSMCs, and activated platelets, suggesting a functional role in the development of atherosclerotic lesions and plaque rupture (6, 18). Endothelial cells themselves are targets of CTGF, leading to proliferation, migration, and in vitro and in vivo angiogenesis (1, 37).

Although hyperlipidemia is now considered a risk factor for the progression of vascular disease, the relationship between increased plasma lipoproteins and endothelial cell dysfunction is poorly defined. Hyperlipidemia can directly or indirectly stimulate the synthesis and release of factors from resident vascular cells, which in turn can influence endothelial cell structure and function in an autocrine or paracrine manner. Therefore, the present study was designed to explore the potential role of LDL in modulating the expression of CTGF in HAECs and to delineate the cellular signaling mechanisms through which this regulation may occur.

**METHODS**

**Endothelial cell culture.** Human aortic endothelial cells (HAECs) were obtained from Cambrex (Walkersville, MD) and cultured using EBM-2 basal serum-free culture medium and EGM-2 SingleQuots growth supplement (2% FBS, 0.1% human epidermal growth factor, 0.04% hydrocortisone, 0.1% vascular endothelial growth factor, 0.4% human fibroblast growth factor-B, 0.1% R3-IGF-1, 0.1% ascorbic acid, 0.1% GA-1000, and 0.1% heparin), which were obtained from Clonetics (Walkersville, MD). HAECs between passages 4 and 9 were used when 70–90% confluent.

**LDL preparation and characterization.** LDL was prepared as previously described (19). Briefly, blood was taken from fasting healthy nondiabetic volunteers into a lipoprotein preservative-antioxidant cocktail (LPPC) containing EDTA (0.1% wt/vol), chloramphenicol (20 μg/ml), gentamicin sulfate (50 μg/ml), e-aminon-caproic acid (0.15%, wt/vol), aprotonin (1 μg/ml) and diethylentriaminepentaacetic acid (1 mM) (final concentrations). Phenylmethylsulfonyl fluoride (PMSF, 20 μg/ml final concentration) was added to plasma to retard proteolysis. All samples were processed at low temperature and in the absence of white light to minimize oxidation. All density solutions were supplemented with LPPC, degassed, and purged with NB38. Plasma density (d) was increased to d = 1.21 g/ml using dried nitrogen and 11 ml layered under d = 1.019 g/ml saline/LPPC. After ultracentrifugation (Beckman VTI50 rotor, 2.5 h, 50,000 rpm, 7°C with slow acceleration and deceleration), the LDL band was harvested by piercing the tube and aspirating into a syringe. LDL isolated by this procedure was free from contamination with apoA-I and albumin. Each LDL preparation was characterized by electrophoresis on 1% agarose gels (Paragon gels, Beckman, Brea, CA). The LDL pools were tested for endotoxin contamination by the Limulus amebocyte lysate (BioWhittaker, Walkersville, MD) according to the manufacturer’s suggestion. These studies were approved by Institutional Review Board of the Medical University of South Carolina, and all subjects signed an informed consent.

**Harvesting of cells and collecting of conditioned media.** Conditioned media samples were collected, centrifuged, and stored at −80°C until required. Endothelial cells treated with LDL were washed twice in ice-cold PBS, scraped in PBS containing 2 mM sodium vanadate, and centrifuged at 3,000 g for 5 min. Pellets were resuspended in 100 μl of lysis buffer (50 mMol/l Tris-HCl, pH 8.0, 150 mMol/l NaCl, 0.02% sodium azide, 0.1% SDS, 1% NP40, 0.5% sodium deoxycholate, 1 mMol/l PMSF, 1.5 μM/ml protease inhibitor cocktail), incubated on ice for 30 min, and centrifuged at maximum speed for 5 min. The supernatant of cell pellet was used as the protein source, and its concentration was determined by a BCA protein assay kit (Pierce, Rockford, IL) by using BSA as a standard protein.

**Western blotting of CTGF and collagen IV.** HAECs at 80% confluence were grown in 1% FBS media without growth factor for 24 h. Endothelial cells were then stimulated with LDL (50 μg/ml) for 24 h in the presence and absence of either a p42/p44 MAPK inhibitor (PD-98059, 40 μM, Calbiochem, La Jolla, CA), a p38 MAPK inhibitor (SB-203580, 10 μM, Calbiochem, La Jolla, CA), and/or the c-Jun NH2-terminal kinase (JNK) inhibitor (SP-600125, 30 μM, A. G. Scientific, San Diego, CA). Soluble protein (20–25 μg) obtained as described above was separated by SDS-PAGE (12% CTGF, 7.5% collagen IV) under reducing conditions and transferred onto nitrocellulose membrane (Bio-Rad, Hercules, CA) with semidy transfer method (Bio-Rad) at 20 V for 50 min. The membranes were immuno-blotted with anti-CTGF polyclonal antibody (1:1,000 dilution, Fibrogen, South San Francisco, CA), anti-collagen IV polyclonal antibody (1:1,000 dilution, Southern Biotechnology, Birmingham, AL), and anti-actin antibody (1:1,000 dilution, Sigma, St. Louis, MO) overnight at 4°C followed by incubating the membranes in a secondary antibody conjugated to horseradish peroxidase (HRP). The immunoreactive bands were visualized using the chemiluminescence reagent Renaissance (NEN Life Science Products, Boston, MA). According to the procedure described by the supplier. Membranes were exposed to Kodak LS film, and bands were measured by densitometry and quantified by Scion Image program (Scion, Frederick, MA).

**MAPK assays.** HAECs at 80% confluence were serum starved by growing them in serum-free media for 24 h. Quiescent endothelial cells were stimulated with LDL (50 μg/ml) for 5 min. Twenty-five to thirty micrograms of protein were analyzed by SDS-PAGE, and the separated proteins were transferred to nitrocellulose membrane with the semitranfer method and immuno-blotted with anti- phospho-p42/44 MAPK polyclonal antibody that detects p42 and p44 MAPK only when activated by phosphorylation at Thr202 and Tyr204 (1:4,000 dilution, Cell Signaling Technology, Beverly, MA), anti-phospho-p38 MAPK antibody that detects p38 only when activated by phosphorylation at Thr180 and Tyr182 (1:1,000 dilution, Cell Signaling Technology), and anti-JNK antibody that detects JNK only when activated by phosphorylation at Thr183 and Tyr185 (1:1,000 dilution, Cell Signaling Technology). Immunoreactive bands were visualized using the chemiluminescence reagent Renaissance, according to the procedure described by the supplier. Membranes were exposed to Kodak LS film, and bands were measured by densitometry and quantified by Scion Image program.

**Promoter activity analysis.** The CTGF promoter containing −823 to +74 region linked to luciferase reporter gene was provided by Dr. Gary Grotendorst. CTGF promoter plasmid DNA was extracted by the alkaline lysis procedure and twice purified on cesium chloride-ethidium bromide gradients. For transient transfections, HAECs were seeded in 12-well plates (750,000 cells/well) in medium containing 2% FBS. The following day, cells at 50–80% confluence were switched to serum-free medium and transiently transfected with 0.5 μg plasmid DNA using FuGENE6 transfection reagent (Roche, Indianapolis, IN) according to the manufacturer’s recommendations. After transfection (16–19 h), HAECs were stimulated with LDL (50 μg/ml) and/or TGF-β (5 ng/ml) for 24 h in the presence and absence of anti-TGF-β neutralizing antibodies (anti-TGF-β NA) (R&D Systems, Minneapolis, MN). Cells were then harvested in 100 μl lysis buffer/well (Promega, Madison, WI), and the protein concentration of each sample was determined using the BCA protein assay kit with BSA as a standard protein. Luciferase activity was measured in different protein concentrations (2.5–30 μg) to determine the linear range of luciferase activity.

**RT-PCR.** RNA was extracted from cells with the use of Qiagen kit (Valencia, CA) according to manufacturer’s protocol. The RNA was converted to cDNA by using MLV-RT (Promega, Madison, WI) according to the manufacturer’s protocol at 37°C for 1 h. The PCR reaction was carried out in 25-μl total volume containing 1×PCR buffer, 200 μM dNTP, 2 ng/μl of each primer, 5 μl of first-strand cDNA, and 1 U of Taq (Qiagen, Valencia, CA). The primers used for
amplification of the human TGF-β1 were 5'-tacctgaacccgtgtgctc-3' and 5'-aacccgtgtgctgctc-3'; those used for human CTGF were 5'-cgggtaataaaaagccttggt-3' and 5'-ttaataaaaagccttggt-3'; those used for human collagen type IV were 5'-agtcgtaataaaaagcttggt-3' and 5'-ggctatagaggtctttacgg-3'; and those used for human β-actin were 5'-gaaactctagagcgcaggttc-3' and 5'-ggaactctagagcgcaggttc-3'. For each target gene, a standard curve was established. This was achieved by performing a series of threefold dilutions of the gene of interest. Negative control was made using the same volume of RNase-free water instead of sample. The master mix was prepared as 2 l of master mix was loaded with a known concentration of LDL (50 μg/ml) into a 25-μl reaction volume of SYBR Green Supermix (Bio-Rad) (12.5 l). For each well, 2 μl of master mix was loaded first, and then 3 μl of sample was added and mixed well to get total reaction volume of 25 μl. For plate setup, SYBR-490 was chosen as fluorophore. The plate was covered with a sheet of optical sealing film. PCR conditions were 95°C for 3 min, followed by 40 cycles of 95°C for 10 s, 60°C for 1 min for CTGF (for β-actin, 58°C for 1 min), then 95°C for 1 min, 55°C for 1 min and 100 cycles of 55°C for 10 s. All of the reactions were done in duplicate. The correlation coefficient was between 0.98 and 1, and PCR efficiency was between 80 and 120%. CTGF mRNA levels were expressed relative to β-actin mRNA.

TGF-β1 protein level determination. HAECs were cultured in six-well plates (9.6 cm²/well). At 80% confluence, cells were serum starved by the changing of serum-free media within 24 h. Cells were then stimulated for 24 h with LDL (50 μg/ml), in the presence or absence of the p42/44mapk inhibitor (PD-98059, 40 μM), the p38mapk inhibitor (SB-203580, 10 μM), and/or the JNK inhibitor (SP-600125, 30 μM) in exactly 1.5 ml of medium. TGF-β1 protein levels were determined by colorimetric enzyme-linked immunosorbent assay kit (ELISA; R&D Systems) in the conditioned media that were activated by 1 N HCl (to measure both active and latent TGF-β1) according to the manufacturer's instructions and expressed as picograms per milliliter.

Statistical analysis. Data are expressed as means ± SE and were analyzed by ANOVA with post hoc corrections (Holm-Sidak method and Dunn's method) and by Student's t-test for paired and unpaired analysis. Differences were considered significant if P < 0.05.

RESULTS

Upregulation of CTGF by LDL. To examine if LDL will modulate the expression of CTGF, HAECs were stimulated with a known concentration of LDL (50 μg/ml) for various times to determine the maximal response. The concentration of LDL used is in the subphysiological range and is above those reportedly necessary to saturate the LDL receptor (10 μg/ml). The results shown in Fig. 1A demonstrate that LDL stimulation resulted in a significant increase in CTGF protein levels at 3 h with a peak response at 24 h (n = 4, P < 0.05).

To determine the optimal concentration of LDL for maximal response, HAECs were stimulated with various concentrations of LDL (0, 10, 25, 50, 75, and 100 μg/ml) for 24 h. The protein levels of CTGF were measured by Western blots using specific anti-CTGF antibodies (1:1,000 dilution). The bar graph (Fig. 1B) represents the intensities of the CTGF bands expressed as percent increase above control. The results shown in Fig. 1 indicate that LDL produced a concentration-dependent increase in the expression of CTGF with a peak response at 50 μg/ml (P < 0.01, n = 4). The protein levels of CTGF were expressed relative to actin protein levels measured in the same
samples. Actin protein levels measured in the same samples were not different between control and LDL-treated cells.

Control experiments were performed to assess the effects of high-density lipoprotein (HDL) on CTGF levels in HAECs. HAECs were stimulated with HDL (50 μg/ml) for 24 h, and the protein levels of CTGF were measured by Western blots and expressed relative to actin protein levels measured in the same samples. The results demonstrated that there was no significant differences in CTGF protein levels between control and HDL-stimulated HAECs (861 ± 116 vs. 819 ± 123 CTGF/actin protein levels; control vs. HDL, respectively, \( P = 0.8074, n = 6 \)).

We next examined if LDL will modulate the expression of CTGF mRNA levels. HAECs were treated with LDL at a concentration of 50 μg/ml for 24 h. RNA levels were extracted from the cells, and the CTGF mRNA levels were determined by RT-PCR. β-Actin mRNA levels were also determined at the same time in the same cell extracts as control. CTGF mRNA levels were expressed relative to β-actin mRNA, and the data are represented in Fig. 2A. Our findings indicate that LDL significantly increased the mRNA levels of CTGF compared with unstimulated cells (\( P < 0.002, n = 12 \)).

The RT-PCR study was repeated using real-time PCR. The CTGF mRNA levels were expressed relative to β-actin mRNA levels. The results demonstrated that LDL stimulation resulted in a significant increase in CTGF mRNA compared with unstimulated cells (54.8 ± 3.69 vs. 45.9 ± 2.88; LDL vs. control, respectively, \( P < 0.01, n = 6 \)). Thus the data generated from real-time PCR support our data generated using RT-PCR. The optimal time for LDL to illicit maximal response on CTGF mRNA expression was also determined. HAECs were stimulated with LDL (50 μg/ml) for various times (3, 6, 24 h), and CTGF mRNA levels were measured by real-time PCR. The results demonstrate that CTGF mRNA levels were increased 3 h after LDL-stimulation with a peak response at 24 h. CTGF mRNA levels for unstimulated control cells were 4.48 ± 0.51 compared with 6.93 ± 0.78 (LDL-3 h), 7.68 ± 0.67 (LDL-6 h), and 9.36 ± 0.21 (LDL-24 h) (\( P < 0.01, n = 3 \)).

To determine whether LDL induces CTGF gene expression at the transcriptional level, CTGF promoter containing 805 bp linked to the luciferase reporter gene (0.5 μg pGL2 plasmid DNA) was transiently transfected into HAECs and stimulated with LDL (50 μg/ml) for 24 h. Luciferase activity was measured in cell lysates normalized for protein concentration. The results shown in Fig. 2B demonstrated that LDL significantly increased the CTGF promoter activity compared with basal activity [14,035 ± 1,701 vs. 7,923 ± 857 relative luciferase units (RLU)/s; LDL vs. control, respectively, \( P < 0.005, n = 4 \)].

We next assessed the effects of LDL on plasmids (pG2) lacking the CTGF promoter region. HAECs were transiently transfected with plasmids lacking the CTGF promoter followed by stimulation with LDL (50 μg/ml) for 24 h. Luciferase activity was measured in cell lysates normalized for protein concentrations. The results demonstrated that no difference in luciferase activity was detected between control and LDL-stimulated cells (22.2 ± 1.7 vs. 21.8 ± 2.0 RLU/s; control vs. LDL-treated cells, respectively, \( P = 0.8727, n = 6 \)).

These findings provide the first evidence that LDL can upregulate the expression of CTGF in HAECs.

**Induction of TGF-β by LDL.** To investigate whether LDL will stimulate the production of TGF-β protein levels, HAECs were treated with LDL (50 μg/ml) for 24 h, and TGF-β protein levels were measured in the conditioned media by ELISA (R&D Systems). The results shown in Fig. 3A demonstrate that LDL treatment resulted in a significant increase in the production of TGF-β compared with unstimulated cells (\( P < 0.005, n = 10 \)).

We next assessed whether LDL will stimulate the expression of TGF-β mRNA levels. HAECs were treated with LDL at a concentration of 50 μg/ml for 24 h, and TGF-β mRNA levels

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**Fig. 2.** LDL increases CTGF mRNA and protein levels in HAECs. A: HAECs were treated with 50 μg/ml of LDL for 24 h. RNA was isolated, and RT-PCR was performed with human CTGF-specific primers. Bar graph represents intensities of CTGF mRNA bands relative to β-actin mRNA expressed as percentage above control. Blots shown are representative of 12 experiments. *\( P < 0.005 \) vs. control. B: HAECs transfected with 0.5 μg plasmid DNA containing the CTGF promoter were treated with 50 μg/ml of LDL for 24 h. Luciferase activity was measured in 5 μg protein, and all values were normalized against expression of plasmid without stimulation. *\( P < 0.05 \) vs. control; \( n = 4 \) experiments.
were determined by RT-PCR and expressed relative to β-actin mRNA levels measured in the same cell extracts. The results shown in Fig. 3B demonstrated that LDL significantly increased the mRNA levels of TGF-β compared with unstimulated HAECs (P < 0.005, n = 12).

Induction of collagen IV by LDL. Collagen I and IV are the predominant matrix proteins of the atherosclerotic plaque of diabetic patients, and collagen IV is the main collagen, produced by endothelial cells. Because matrix deposition is the hallmark of atherosclerosis, it is crucial to determine whether LDL will influence the deposition of matrix proteins in cells prone to injury. In this regard, we examined if LDL will modulate the expression of collagen IV mRNA as well as protein levels. HAECs were treated with LDL at a concentration of 50 μg/ml for 24 h. RNA levels were extracted from the cells, and the collagen IV mRNA levels were determined by RT-PCR. β-Actin mRNA levels were also determined at the same time in the same cell extracts as control. Collagen IV mRNA levels were expressed relative to β-actin mRNA, and the data are represented in Fig. 4A. Our findings indicate that LDL significantly increased the mRNA levels of collagen IV compared with unstimulated cells (P < 0.0005, n = 12). Collagen IV protein levels in cell extracts were measured by Western blots by using specific anti-collagen IV antibodies (1:1,000 dilution). Collagen IV protein levels were expressed as percentage of control, and the data are represented in Fig. 4B. The results demonstrate that LDL significantly increased the protein levels of collagen IV compared with control (P < 0.001, n = 13). The levels of the structural protein actin were also measured in the same cell extracts as control, to ensure equal loading of the samples into the gel, and were not different between control and LDL-treated cells.

The levels of collagen IV protein in the conditioned media were also measured. The results shown in the Fig. 4C demonstrate that LDL-treated HAECs produced a significantly higher level of collagen IV in their media than unstimulated cells (2,264.5.6 ± 274.3 vs. 1,310.8 ± 179; LDL vs. control, respectively, P < 0.005, n = 22). Induction of CTGF by TGF-β. To explore the cellular mechanisms through which LDL regulates the expression of CTGF in HAECs, we examined whether CTGF is a downstream target for regulation by TGF-β. The results shown in Fig. 5A demonstrate that HAECs stimulated with TGF-β (5 ng/ml) for 24 h significantly increased the promoter activity of CTGF compared with unstimulated cells (16,265.13 ± 2,605.7 vs. 7,923.25 ± 856.8 RLU/s; TGF-β vs. control, respectively, P < 0.005, n = 4).

The levels of the structural protein actin were also measured in the same cell extracts as control to ensure equal loading of the samples into the gel. The results demonstrate that TGF-β resulted in a concentration-dependent increase in the expression of CTGF (P < 0.005, n = 10). This increase in CTGF protein levels induced by TGF-β was eliminated in the presence of anti-TGF-β NA (Fig. 5B). Actin protein levels were not different between control and TGF-β-treated cells. These findings indicate that TGF-β is upstream of CTGF and may be involved in modulating the expression of CTGF.
significant increase in CTGF protein levels ($P < 0.005$ vs. control, $n = 4$). This increase in CTGF in response to LDL was significantly inhibited by the anti-TGF-β NA ($P < 0.005$ vs. LDL). Anti-TGF-β NA did not significantly influence the basal protein levels of CTGF. Mouse nonimmune serum had no significant effect on LDL-induced CTGF expression in HAECs (Fig. 6C). These findings suggest that TGF-β signaling contributes to LDL stimulation of CTGF expression in endothelial cells. Our findings are in support of previous reports showing that LDL stimulates collagen expression in human mesangial cells via activation of TGF-β (27).

Phosphorylation of MAPK pathway by LDL. To determine whether LDL will activate members of the MAPK family, HAECs were treated with LDL (50 μg/ml) for 5 min. Cytosolic proteins were analyzed by SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with either anti-phospho-MAPK or anti-MAPK antibodies (Fig. 4). LDL stimulation of MAPK activity was detected in a dose-dependent manner. Inhibition of MAPK activity by a specific inhibitor (PD98059) significantly reduced LDL-induced CTGF expression (Fig. 5). These findings suggest that LDL stimulation of CTGF expression is mediated through the MAPK pathway.

Fig. 4. LDL stimulates collagen IV mRNA and protein levels in HAEC. A: HAECs were treated with 50 μg/ml of LDL for 24 h, and collagen IV mRNA levels were measured by RT-PCR. Bar graph represents intensities of collagen IV mRNA bands relative to β-actin mRNA expressed as percentage above control. Blots shown are representative of 12 experiments. *$P < 0.005$ vs. control. $B$: HAECs were stimulated with 50 μg/ml of LDL for 24 h. Cell proteins were separated by SDS-PAGE (7.5%) and immunoblotted with an antibody against collagen IV (1:1,000) and actin (1:1,000). Bar graph represents intensities of collagen IV bands relative to actin expressed as percentage above control. Blots shown are representative of 13 experiments. *$P < 0.005$ vs. control. C: quiescent HAECs were stimulated with 50 μg/ml of LDL for 24 h. Conditioned media from cells were collected. Equal volumes (28 μl/lane) of conditioned media were separated by SDS-PAGE (7.5%) and immunoblotted with an antibody against collagen IV (1:1,000). Bar graph represents intensities of collagen IV bands expressed as percentage above control. Blots shown are representative of 22 experiments. *$P < 0.001$ vs. control.

Fig. 5. Induction of CTGF by TGF-β. A: HAECs transfected with 0.5 μg plasmid DNA containing the CTGF promoter were treated with 5 ng/ml of TGF-β for 24 h. Luciferase activity was measured in 5 μg protein, and all values were normalized against expression of plasmid without stimulation. *$P < 0.05$ vs. control, $n = 4$ experiments. B: HAECs were treated with 10 and 20 ng/ml of TGF-β for 24 h in presence or absence of 5 μg/ml of neutralizing anti-TGF-β antibody (TGF-β NA). Cell proteins were separated by SDS-PAGE (12%) and immunoblotted with an antibody against CTGF (1:1,000) and actin (1:1,000). Bar graph represents intensities of CTGF bands relative to actin expressed as percentage above control. Blots shown are representative of 10 separate experiments. *$P < 0.005$ vs. control. #P < 0.001 vs. TGF-β1.
Phospho-p42/p44 MAPK antibodies (1:4000 dilution) or anti-phospho-p38 MAPK (1:1000 dilution) and/or anti-phospho-JNK antibodies (1:1000 dilution). The membranes were also immunoblotted with total nonphosphorylated p42/p44 MAPK, p38 MAPK, and JNK antibodies to ensure equal amounts of MAPK loaded into the gels. The results shown in Fig. 7, A–C, demonstrate that LDL significantly increased the phosphorylation of p42/p44 MAPK (P < 0.0001, n = 12), p38 MAPK (P < 0.0001, n = 12), and of JNK (P < 0.0002, n = 12). Total p42/p44 MAPK, p38 MAPK, and JNK were not different between control and LDL-treated cells.

Role of MAPK pathway in LDL-induced TGF-β production. To explore whether the MAPK pathway modulates the production of TGF-β in response to LDL, we measured TGF-β protein levels in HAECs treated with LDL (50 μg/ml) for 24 h in the presence and absence of membrane-permeable inhibitors of p42/p44 MAPK (PD-98059, 40 μM), p38 MAPK (SB-203580, 10 μM), and JNK (SP-600125, 30 μM). The results shown in Fig. 6. LDL induces CTGF expression via autocrine activation of TGF-β. A: HAECs transfected with 0.5 μg plasmid DNA containing the CTGF promoter were treated with 50 μg/ml of LDL for 24 h in the presence and absence of anti-TGF-β NA (5 and 10 μg/ml). Luciferase activity was measured in 5 μg protein, and all values were normalized against expression of plasmid without stimulation. *P < 0.002 vs. control, #P < 0.03 vs. LDL; n = 6 experiments. B: HAECs were stimulated with 50 μg/ml of LDL for 24 h in presence or absence of 5 and 10 μg/ml of anti-TGF-β NA. Equal amounts of proteins were resolved on SDS-PAGE (12%) and immunoblotted with an antibody against CTGF (1:1000) and actin (1:1,000). Bar graph represents intensities of CTGF bands relative to actin expressed as percentage above control. Blots shown are representative of 4 experiments. *P < 0.005 vs. control, #P < 0.05 vs. LDL. C: HAECs were stimulated with LDL (50 μg/ml) for 24 h in the presence and absence of mouse nonimmune serum (NS, 5 μg/ml). Bar graph represents intensities of CTGF bands relative to actin expressed as percentage above control. Blots shown are representative of 4 experiments. *P < 0.05 vs. control.
demonstrate that TGF-β protein levels were modestly reduced by the ERK inhibitor PD-98059 (P < 0.005, n = 8). On the other hand, the JNK inhibitor SP-600125 markedly reduced the increased production of TGF-β protein levels in response to LDL (P < 0.005, n = 8), whereas selective inhibition of p38 MAPK by SB-203580 did not have a significant effect. These findings demonstrate that LDL utilizes the ERK and JNK pathway to modulate the production of TGF-β in HAECs.

Role of MAPK in LDL-induced CTGF production. To explore whether the MAPK pathway modulates the production of CTGF in response to LDL, we measured CTGF protein levels in HAECs treated with LDL (50 μg/ml) for 24 h in the presence and absence of membrane-permeable inhibitors of p44/42 MAPK (PD-98059, 40 μM), p38 MAPK (SB-203580, 10 μM), and JNK (SP-600125, 30 μM). The results shown in Fig. 9 demonstrate that LDL once again produced a significant increase in CTGF protein levels compared with unstimulated cells. However, in the presence of the p44/42 MAPK inhibitor PD-98059 and/or the JNK inhibitor SP-600125, the increase in CTGF protein levels in response to LDL was significantly suppressed (P < 0.01, n = 7–8). On the other hand, inhibition of the p38 MAPK by SB-203580 did not alter the expression of CTGF in response to LDL. Actin protein levels measured at the same time and in the same cell extracts were not significantly different among the groups.

This is the first demonstration that the induction of CTGF by LDL in endothelial cells is mediated via activation of the MAPK pathway.
24 h. The results shown in Fig. 10 demonstrate that LDL produced a significant increase in collagen IV protein levels in the conditioned media compared with unstimulated cells. This increase in collagen IV protein levels was significantly reduced by the JNK inhibitor SP-600125 (\(P < 0.05, n = 6\)). However, selective inhibition of p44/p42 mAPK by PD-98059 and of p38 mAPK by SB-203580 did not significantly alter the increased production of collagen IV protein levels in response to LDL stimulation. These findings demonstrate that LDL utilizes the JNK pathway to modulate the production of collagen IV in HAECs.

**DISCUSSION**

In the present study we have demonstrated that LDL exerts a significant effect on the expression of CTGF and collagen IV in endothelial cells. We have shown that LDL increases CTGF promoter activity, the mRNA and the protein levels of CTGF,
TGF-β, and collagen IV in endothelial cells. This effect of LDL was mediated via activation of the MAPK pathway. The risk for atherosclerosis is augmented when plasma LDL-cholesterol levels are increased, and many interventional studies have demonstrated that pharmacological treatment of coronary-prone patients with lipid-lowering agents reduces coronary events and total mortality (14, 36, 38). An important early event in the initiation of atherosclerosis is the increased uptake of monocytes into the intima where they differentiate into macrophages and ingest modified forms of LDL to become foamy macrophages, which give rise to “fatty streaks,” the precursor lesion that subsequently leads to development of atherosclerosis (11, 21, 23, 32). Consequently, much attention is now focused on understanding the etiology of the fatty streak and the mechanisms by which LDL can affect components of the atherosclerotic process. However, much less is known about the cellular and molecular mechanisms by which native LDL activates endothelial cells to influence atherogenesis.

Although hyperlipidemia is now considered a risk factor for the progression of atherosclerosis, the initiating and sustaining signaling pathways that link hyperlipidemia to atherosclerosis are not fully realized. Accumulation of LDL within the intima may activate endothelial cells to produce various cytokines and growth factors that may alter endothelial cell behavior by inducing cell proliferation, migration, invasive capacity, and tubulogenesis. In this regard our findings demonstrate that the protein levels of TGF-β, CTGF, and collagen IV were induced in endothelial cells in response to LDL challenge. Inhibition of TGF-β activity with neutralizing antibodies partially prevented the rise in CTGF, thus demonstrating that the increase in CTGF protein levels in response to LDL was mediated via activation of TGF-β-dependent and -independent mechanisms. Even though TGF-β has long been regarded as a major driving force in many progressive fibrotic diseases, attention has recently focused on the role of CTGF as a profibrotic factor (25). CTGF, a newly described factor that promotes ECM deposition and fibrosis in many tissues, appears to act downstream of TGF-β to induce ECM production (33). Several studies demonstrated CTGF is an important mediator in the pathogenesis of atherosclerosis (2, 15, 29, 31). CTGF in endothelial cells is upregulated by lysophosphatidic acid, sphingosine-1-phosphate, and platelets (29). However, in the present study we demonstrate for the first time that LDL can directly and acutely upregulate CTGF protein levels in endothelial cells. Our findings indicate that the upregulation of CTGF by LDL could be mediated through dependent or independent mechanisms involving autocrine activation of TGF-β. It is widely accepted that LDL stimulation results in the activation of members of the MAPK family, and activation of this pathway is known to be important in regulating gene expression and endothelial cell growth and function (30). The MAPKs are a family of serine-threonine protein kinases that are activated in response to a variety of extracellular stimuli. ERK (p42/44mapk), p38mapk, and JNK constitute three major subfamilies of MAPK that appear to mediate cellular responses, including proliferation, differentiation, and apoptosis (43). ERK plays a major role in cell proliferation and differentiation, as well as in survival mediated by various growth factors (24). On the other hand, p38mapk and JNK are activated by various inflammatory cytokines and environmental stressors, and they play important roles in apoptosis and cytokine production (24).

To elucidate which member of the MAPK family may be responsible for the LDL-induced increase in CTGF and collagen IV, we elected to study the role of p42/44mapk, p38mapk, and JNK in HAECs. Our findings indicate that inhibition of JNK by SP-600125 suppressed the expression of TGF-β, CTGF, and collagen IV levels in response to LDL stimulation, whereas inhibition of the p42/44mapk by PD-98059 resulted in suppression of LDL-induced expression of CTGF. On the other hand, blockade of the p38mapk pathway by SB-203580 did not significantly alter the expression of TGF-β, CTGF, or collagen IV in response to LDL. This finding implicates JNK as a key player in modulating the signals through which LDL promotes TGF-β, CTGF, and collagen IV expression in HAECs. Furthermore, it is of interest to note that inhibition of basal JNK activity also reduced the basal production of TGF-β, thus implicating a key role for JNK kinase in modulating the production of TGF-β in HAECs. Other studies have implicated JNK in TGF-β-induced CTGF mRNA expression in human lung fibroblasts, whereas p38mapk and p42/44mapk pathways were implicated in TGF-β-induced extracellular matrix deposition (8, 16, 41). We also found JNK is a key player in modulating the expression of TGF-β, CTGF, and collagen I in response to LDL stimulation in rat mesangial cells (38).

Although the initiating and sustaining signals that link LDL to CTGF expression are not yet fully defined, several possibilities may exist. Our data demonstrate that LDL stimulates JNK activation, and JNK has been shown to bind and phosphorylate the DNA binding protein c-Jun and increase its transcriptional activity (24). c-Jun is a component of the activator protein-1 (AP-1) transcription complex, which is an important regulator of gene expression (20). In this regard, the autinduction of TGF-β has been shown to be mediated via activation of the AP-1 complex (22). Once activated, TGF-β can induce CTGF expression via the Smad pathway (7). In fact, a Smad response element is present on the promoter of the CTGF gene (26).

In summary, we demonstrate that the expression of CTGF and collagen IV in endothelial cells are upregulated by LDL and that this regulation is mediated via autocrine activation of TGF-β. The results also implicate JNK as a key player in modulating the expression of TGF-β, CTGF, and collagen IV in response to LDL stimulation. The data also point to a potential novel pathway through which lipoproteins may promote atherosclerotic injury.

ACKNOWLEDGMENTS

We thank Dr. William Usinger (FibroGen, South San Francisco, CA) for the generous gift of anti-CTGF antibodies.

GRANTS

This work was supported by National Institutes of Health Grants DK-46543 and HL-55782 (to A. A. Jaffa).

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