High Density Lipoproteins Stimulate the Production and Secretion of Endothelin-1 from Cultured Bovine Aortic Endothelial Cells

Ren-Ming Hu,* Mei-Yu Chuang,‡ Bruce Prins,* Moti L. Kashyap,‡ Harrison J. L. Frank,* Ali Pedram,* and Ellis R. Levin*§
**Divisions of Endocrinology and Gerontology and ‡the Cholesterol Center, Veterans Affairs Medical Center, Long Beach, California 90822; and §Departments of Medicine and Pharmacology, University of California, Irvine, Irvine California 92717*

Abstract

The concentration of HDL in the blood inversely correlates with the incidence of cardiovascular disease, probably related to the ability of these lipoproteins to efflux cholesterol from vascular cells. It is also possible that HDL affect the production or action of vasoactive peptides implicated in the development of vascular diseases. Therefore, we determined the effects of human HDL on the production and secretion of endothelin-1 (ET-1) from cultured bovine aortic endothelial cells. HDL produced a highly significant stimulation of endothelin secretion (maximum 240% of control), even at very low levels of lipoproteins (1 μg/ml). HDL also stimulated the translation of ET-1 by twofold in the bovine aortic endothelial cells. In contrast, HDL had no significant effect on steady state mRNA levels, transcript degradation, or transcription. Stimulation of ET-1 secretion by HDL was dependent on protein kinase C activation. Purified apo A-I, the major apoprotein of HDL, increased ET-1 secretion and translation ~ 85% as potently as HDL. Our results indicate that low concentrations of human HDL strongly stimulate the production of ET-1, a powerful vasoconstrictor and mitogen for the vascular smooth muscle cell. We propose that HDL may participate in the regulation of vasomotor tone through this potentially important effect in the vasculature. (*J. Clin. Invest.* 1994. 93:1056–1062.) Key words: endothelin • high density lipoprotein • apolipoprotein A-I • transcription • translation

Introduction

The development of atherosclerosis is a complex process involving the production and action of proteins and other factors. Early events include the disruption of the endothelium, at which time lipid-laden fibrous plaque accumulates within the endothelial lining of arteries, and smooth muscle cells proliferate and migrate into the intima (1). The process of incorporation of cholesterol into the plaque is opposed by the efflux promoted by HDL (2). It is believed that the strong association between high plasma levels of HDL and a decreased incidence of cardiovascular disease is related to this ability of HDL to effect reverse cholesterol transport (3, 4). However, it is also conceivable that HDL might interact with important vascular hormones or growth factors to influence the development of atherosclerosis or to modulate other aspects of blood vessel function. Some of these interactions may actually promote atherosclerosis. For instance, HDL has been shown to act as a strong mitogen for vascular cells, particularly in conjunction with other growth factors (5, 6). Also, insulin, IGF-1 (7), and PDGF (8) can decrease HDL-mediated efflux of cholesterol from cells. These functions provide a mechanism whereby protein growth factors can interact with HDL and theoretically potentiate the atherosclerotic process. One recently discovered peptide produced in endothelial cells, endothelin-1 (ET-1),[1] is a powerful endogenous vasoconstrictor and mitogen for the vascular smooth muscle cell (9, 10). ET-1 has been implicated in the pathogenesis of both acute and chronic vascular disease (10–12) including the development of atherosclerosis (13). Thus, it would be important to determine possible interactions between ET-1 and HDL to better understand the dynamic events regulating vasomotor tone and the development of vascular disease. In these studies, we determined whether human HDL can affect the production and secretion of ET-1, what part of the HDL moiety might be responsible, and the mechanism of HDL action.

Methods

Endothelial cell cultures and experiments. Bovine aortic endothelial cell (BAEC) cultures were prepared as described previously (14, 15). Cells were seeded at a density of 77,000 cells/cm^2 on 100-mm or 6-well culture plates, passaged once after treatment with trypsin, replated and cultured in DME with 10% FBS, and then used for experiments ~ 1 wk after cell preparation. The cells displayed the expected morphologic characteristics of endothelial cells, and virtually all cells showed positive fluorescence with an antibody to Factor VIII, indicating the homogeneity of the cultures. The cultures were devoid of any cells having the appearance of smooth muscle cells.

For experiments, subconfluent cells were incubated in DME media without serum for various times (time course) up to 8 h, in the presence or absence of different concentrations (0.1–50 μg/ml) of HDL or apo A-I protein (Sigma Immunochemicals, St. Louis, MO). The media was frozen for subsequent radioimmunoassay of ET-1, and the RNA was extracted from both control (no HDL) and the various experimental plates (four per condition) as described previously (14, 15). For assessment of protein kinase C (PKC), cells were incubated with the PKC inhibitor (16), calphostin C, 50 nM, for 30 min before HDL addition. In further experiments, the BAEC were incubated with the phorbol ester, PMA (1 μM), for 24 h (to downregulate cytosolic PKC) before HDL addition.

HDL purification. Fasting human serum was subjected to sequential flotation ultracentrifugation (17), and a narrow density cut of HDL_3 (d = 1.12–1.21 g/ml) was further processed. The preparation was then dialyzed against 0.1 M PBS (pH 7.4) and 0.5 M NaCl overnight at 4°C, with two changes of dialysate. The purity of the resulting HDL was determined by SDS-PAGE which showed that > 80% of the

Address correspondence to Ellis R. Levin, M.D., Medical Service (111-I), Long Beach Veterans Hospital, 5901 East 7th Street, Long Beach, CA 90822.

Received for publication 12 July 1993 and in revised form 17 November 1993.

1. *Abbreviations used in this paper:* BAEC, bovine aortic endothelial cells; ET, endothelin; PKC, protein kinase C.

total apoprotein was apo A-I, with minor apo A-II, apo E, and apo C, but no apo B present. The isolated HDL showed a single α-migrating lipoprotein band by agarose gel electrophoresis. HDL protein was then used for all experiments.

ET radioimmunoassay. ET immunoreactivity was measured by a sensitive double-antibody, nonequilibrium assay, as described previously (14, 15). The sensitivity of the assay was 2–3 pg/tube, and the intra- and interassay coefficients of variation were always < 10%.

S1 nuclease protection. The extracted RNA was hybridized with a ^{32}P-labeled cRNA probe made from an EcoRI template of a human cDNA for ET-1 (provided by Dr. Ken Bloch, Harvard Medical School) (18). The cDNA was subcloned into a Hind3-EcoR1 orientation in PBS, and antisense and sense cRNA probes were promoted using T_7 and 3,5,3′-triiodothyronine RNA polymerase, respectively. The cRNA probe spans parts of exons 5 and 4 from nucleotide 718 to 1182 of the human ET-1 gene (18), but because of the different sequences in the human and bovine ET-1 mRNAs, a transcript of 196 bases was protected. Hybridization, S1 nuclease digestion, and electrophoretic separation were carried out as described previously (14, 15). A HindIII-digested and ^{32}P-labeled cRNA for H-*ras* served as an RNA-loading standardization probe. The gel was opposed to film with intensifying screens for 24 h, and the autoradiographic bands were compared by laser densitometry (LKB Instruments Inc., Bromma, Sweden). Sense probes produced no hybridization.

Nuclear runoff transcription. BAEC were grown in DME with 10% FBS. For experiments, BAEC were incubated in the presence of HDL 50 μg/ml for 2, 4, and 6 h, while control cells were incubated in the absence of HDL. We also incubated cells for 4 h in the presence or absence (control) of HDL or the phorbol ester PMA, 10^{-7} M, a known stimulator of ET-1 transcription. The cells from each experimental treatment or control were washed and then were lysed in NP-40 lysis buffer. The nuclei were isolated after centrifugation and resuspension in NP-40 buffer, followed by freezing in glycerol storage buffer until use. Transcription studies were carried out as we described previously (15): the thawed nuclei (5×10^7 cells) were added to equal volume reaction buffer with 4 mM ATP, GTP, and CTP plus 10 μl of [^{32}P]-UTP. After a 30-min incubation, DNA was digested with DNase, followed by proteinase K digestion. The newly formed, ^{32}P-labeled RNA was then purified and transferred onto GFA filters (Whatman Inc., Clifton, NJ), and an aliquot was counted. To detect the presence of ET-1 RNA and the effects of HDL or PMA, equal counts of labeled nuclear RNA were hybridized with the cDNA for ET-1, and were spotted onto nitrocellulose filters (using a slot blot apparatus). Comparison was made using a cDNA for β-actin. After 36 h of hybridization, the filters were washed, were RNase treated, and then were exposed to x-ray film for 1–3 d.

ET-1 mRNA half-life studies. Endothelial cells were incubated in the presence of actinomycin D, 10^{-6} M, for 15, 30, or 60 min, at which times total RNA was extracted. In parallel, daughter cells were coincubated with actinomycin D plus ET-1, 10^{-6} M, for identical times. After solution hybridization and S1 nuclease digestion, the hybridized RNA samples were separated on denaturing polyacrylamide gel, and a half-life for the ET-1 transcript was calculated from the density of the autoradiographic bands at each time point, in the presence or absence of HDL. Data from three experiments were combined. The times selected were based upon the known half-life of ET-1 in BAEC, which we determined previously (14).

In vivo translation studies. BAEC were cultured for 10 d, and then were incubated in methionine-free DME medium with dialyzed 10% FBS (or no FBS in some experiments) for 1 h before experimentation (14). The cells were then incubated in the presence or absence of HDL 50 or 1 μg/ml or apo A-I 50 μg/ml, and 250 μCi of [^{35}S]methionine for 2 h. The media were aspirated and saved, and the cells were then washed free of unincorporated labeled methionine and were lysed in a buffer containing 1% Triton X-100/1% bovine hemoglobin/1 mM iodoacetamide and aprotinin (protease inhibitor) 0.2 U for 1 h at 4°C. The lysate and secretion media were then precleared with normal rabbit serum. Specific labeled ET-1 protein was immunoprecipitated using polyclonal antibody to ET-1 and anti-IgG second antibody (14). Antibody which was preabsorbed with ET-1, 10^{-6} M, for 12 h before use in the immunoprecipitation procedure served as a specificity control. The immunoprecipitated protein was then solubilized in SDS sample buffer and was heated to 100°C for 5 min. The labeled protein was then resolved on a 4% stacking/10% spacing/16.5% separating polyacrylamide gel and was separated by electrophoresis at 30–50 mA and constant voltage using a tricine buffer system. The size of the protein bands was compared with molecular weight markers resolved under the same circumstances. The gel was then stained and destained and was subjected to fluorography and then autoradiography for 10–21 d. Each translation experiment was performed four times.

Statistics. Data from secretion studies were combined (n = 11–12 wells/condition) and then were analyzed by calculating a mean and standard error for each treatment or group. Data from the different conditions were compared by ANOVA; a multiple range test (Scheffe's) was used for significant F values ($P < 0.05$). All secretion studies were carried out at least three times. RNA comparisons were quantified by laser densitometry of autoradiographs, and data were normalized for RNA loading by creating a ratio of the density of the experimental RNA hybridized with the ET-1 probe, divided by the same amount of RNA hybridized with H-*ras*. A ratio was then established by comparing normalized experimental RNA with normalized control RNA which was extracted from nontreated endothelial cells. A value of 1 was arbitrarily assigned to the control. This resulted in values expressing the relative densities of the experimental conditions compared with the control. Protein bands were also compared by laser densitometry.

Results

ET-1 secretion. HDL, 50 μg/ml, caused a significant increase in the secretion of ET-1 which was first observed by 2 h and persisted for the entire 8 h of the in vitro experiments (Fig. 1). HDL caused a peak increase of secretion at 4–8 h, significantly greater than BAEC incubated in the absence of HDL (mean control secretion at 4 h 492±70 [SEM] pg/ml; mean HDL-incubated secretion 1,028±23 pg/ml, n = 11). In a dose-related fashion, HDL maximally stimulated ET-1 by more than two-fold at the highest concentrations used (50 and 100 μg/ml) and significantly increased ET-1 secretion at a concentration as low as 1 μg/ml in studies carried out over 4 h of incubation (Fig. 1 *B*). The similar stimulation of ET-1 secretion at 50 and 100 μg/ml of HDL suggests that the process is receptor mediated and saturable at ~ 50 μg/ml. We previously determined that the ET-1 immunoreactivity detected by radioimmunoassay in the secretion media from the cultured BAEC is ET-1, by HPLC and immunoassay criteria (15).

The secretion of ET-1 was also significantly stimulated by the major apoprotein moiety of HDL, apo A-I (Fig. 1 *C*). At 50 μg/ml, apo A-I significantly stimulated the secretion of ET-1 from the BAEC (mean control secretion 642±21 pg/ml; mean apo A-I–stimulated secretion 1,061±18 pg/ml) which was 83% comparable with the stimulation caused by HDL at this same concentration (mean HDL-stimulated secretion 1,271±12 pg/ml, n = 11 from three experiments combined). This suggests that this apoprotein was mainly responsible for the stimulation of ET-1 secretion by HDL. This conclusion is supported by our finding that the purified HDL we prepared from human plasma contained predominantly apo A-I.

Translation studies. In vivo translation studies within the BAEC revealed the intracellular production of prepro–ET-1 (24,000 M_r), and a large molecular weight (18,000) form of ET which probably represents a novel intermediate, processed

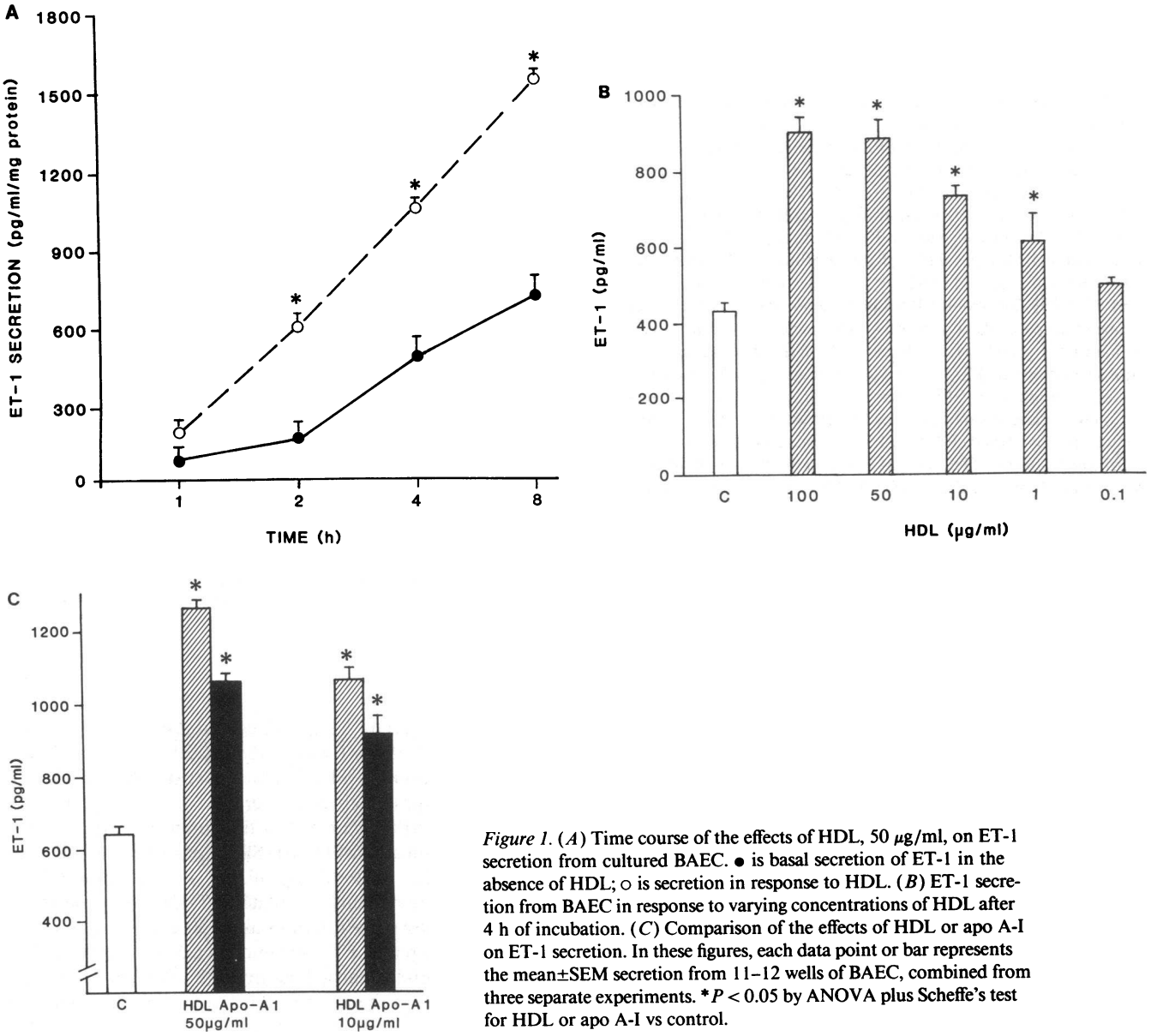

Figure 1. (*A*) Time course of the effects of HDL, 50 μg/ml, on ET-1 secretion from cultured BAEC. ● is basal secretion of ET-1 in the absence of HDL; ○ is secretion in response to HDL. (*B*) ET-1 secretion from BAEC in response to varying concentrations of HDL after 4 h of incubation. (*C*) Comparison of the effects of HDL or apo A-I on ET-1 secretion. In these figures, each data point or bar represents the mean±SEM secretion from 11–12 wells of BAEC, combined from three separate experiments. $*P < 0.05$ by ANOVA plus Scheffe's test for HDL or apo A-I vs control.

form of prepro–ET-1, which we described previously (14) (Fig. 2). We did not see Big endothelin (pro–ET-1) or the fully processed, 21–amino acid ET-1 (2,500 M_r) in the cell lysate. In contrast, the secretion media contained both Big ET-1 and ET-1, forms which are known to be secreted and which circulate in plasma. This indicates that the processing of the preprohormone to the pro-(Big) protein is likely to occur in the cytoplasm of the BAEC. This is followed rapidly by the partial processing of the prohormone to the mature ET-1 peptide in our cell culture system and probably occurs just within the cytosol or at the cell membrane just before secretion of ET-1. In vivo, serum proteases are felt to contribute to the processing of the prohormone to the mature, 21–amino acid ET-1. We found predominantly the 21–amino acid ET-1 in our cell secretion media, which lacks serum-processing enzymes. This suggests that the majority of processing of the precursor forms is accomplished by the endothelial cell itself.

The moieties detected are consistent with the processing of prepro–ET-1 from BAEC induced by atrial natriuretic peptide (14), except that the antibody we used in these studies detects all three described forms of ET-1. Most importantly, we found that HDL, in dose-related fashion, significantly stimulated the translation of all three forms of the ET-1 peptide (Fig. 2). For instance, HDL, at a concentration of 50 μg/ml, caused a 2.13±0.11-fold increase over the control production of prepro–ET-1 (density of bands). The immunoprecipitated bands were identified as ET-1 or its precursors; this was established by using an antibody which was preabsorbed with ET-1 and hence could no longer bind and precipitate ET-1–related protein in the cell lysate or secretion media. In our studies, the preabsorbed antibody precipitated the same bands in control cells < 8% as well as nonabsorbed antibody (Fig. 2 *A* or *B*, lane *1* vs *5*). Equal amounts of apo A-I were 80±4% as effective as HDL in increasing ET-1 production, by densitometry (Fig. 2). As summated in Table I, HDL caused a twofold increase in both precursor ET-1 (cell lysate) or the 21–amino acid, ET-1 pep-

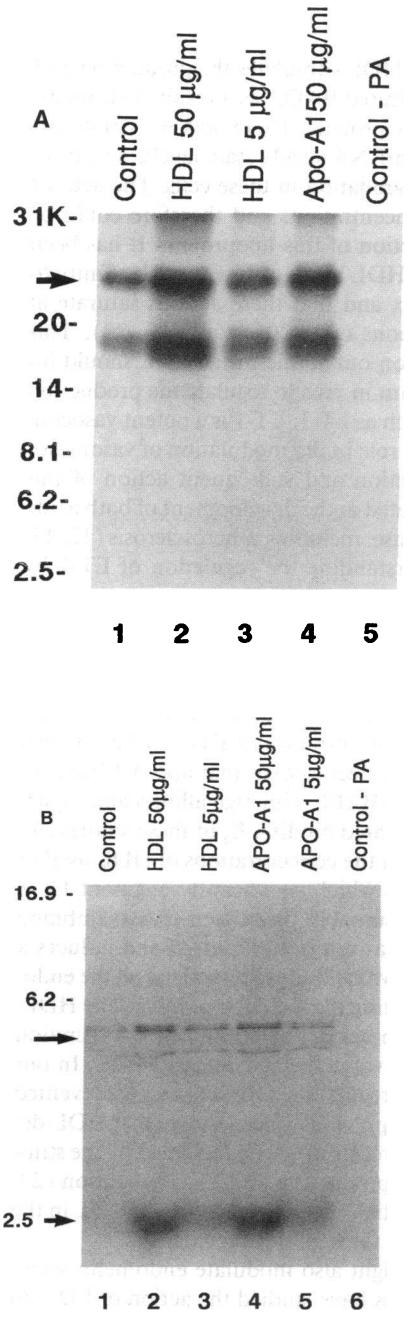

Figure 2. Stimulation of ET-1 translation by HDL or apo A-I. Cultured BAEC were incubated with 250 µCi of [^{35}S]methionine for 2 h in the absence (*Control*) or presence of HDL 50 or 5 µg/ml or apo A-I 50 µg/ml. The media were then saved, and the cells were lysed and were precleared with normal rabbit serum, then ET-1–related protein was immunoprecipitated with polyclonal antibody to ET-1 and anti-IgG antibody. Labeled protein was then separated by SDS-PAGE on a 16.5% separating gel using a tricine buffer system. Molecular weight markers resolved under the same conditions are noted. (*A*) Lanes *1–5* represent cell lysate from control BAEC and cells incubated with HDL at 50 or 5 µg/ml, apo A-I 50 µg/ml, or control cell lysate immunoprecipitated with preabsorbed antibody, respectively. The arrow indicates a labeled protein of ~ 24,000 mol wt, compatible with prepro–ET-1. (*B*) Lanes *1–6* represent, respectively, incubation media from control BAEC, cells incubated with HDL at the two concentrations, apo A-I 50 and 5 µg/ml, or media from control BAEC immunoprecipitated with preabsorbed antibody. The arrows indicate labeled proteins of ~ 4,300 and 2,500 mol wt, compatible with Big ET-1 and ET-1. The experiment was repeated an additional three times, and autoradiography was performed three times. The density values or comparisons in the text are derived from the mean±SEM from three experiments combined.

Table I. Incorporation of [^{35}S]Methionine into Immunoprecipitated, Newly Translated, ET-1–related Protein

	Experimental condition	Incorporated (cpm)
		% of control
Intracellular lysate	Control	100
	HDL 50 µg/ml	211±5*
	HDL 5 µg/ml	137±11*
	Apo A-I 50 µg/ml	180±9*
	Control-preabs	11±2*
Secretion media	Control	100
	HDL 50 µg/ml	200±9*
	HDL 5 µg/ml	142±20*
	Apo A-I 50 µg/ml	186±5*
	Control-preabs	12±3*

Each value is the mean±SEM of data from four separate experiments, results combined. The counts per minute for each experimental condition are expressed as percentage of [^{35}S]methionine incorporation into immunprecipitable ET-1–related proteins from cells that were incubated without lipoprotein (*Control*). * $P < 0.05$ by ANOVA plus Scheffe's test for condition vs control. The control-preabs are counts from control cells, the lysate or secretion media immunoprecipitated with antibody preabsorbed overnight with ET-1 protein.

tide production (secretion media), compared with basal synthesis. Apo A-I was also potent in this regard, stimulating increased prepro and ET-1 protein synthesis by 85±5 (lysate) and 93±4% (secretion) comparably with HDL (Table I). This indicates that apo A-I probably is responsible for the great majority of this action of HDL.

Messenger RNA studies. Incubation of the BAEC for various amounts of time over 6 h induced no significant change in the steady state mRNA levels, shown by protection assay (Fig. 3 *A, top*). There was a slight increase in ET-1 message of only 22±4% at 4 h. Because steady state mRNA expression reflects both transcription and transcript degradation, we examined both of these issues. The half-life of the ET-1 mRNA, which was ~ 15 min, was not altered by HDL (Fig. 3 *B*). From the nuclear run-on studies, HDL had no detectable effect on ET-1 transcription at either 2 or 6 h (data not shown). HDL stimulated ET-1 transcription maximally by only 26±6% at 4 h, compared with control cells and after normalization for RNA loading (Fig. 3 *C*). By comparison, phorbol ester induced a 2.1±0.4-fold increase in ET-1 transcription at 4 h (Fig. 3 *C*). We conclude that the ability of HDL to increase the production of ET-1 resides mainly at a posttranscriptional level, specifically by inducing increased translation, followed by increased secretion of the peptide.

PKC. Incubation of the BAEC with the relatively specific PKC inhibitor, calphostin C, 50 nM, completely reversed the increased secretion of ET-1 caused by HDL, 10 µg/ml (Fig. 4). After 4 h of incubation, HDL stimulated the secretion of ET-1 from control levels of 352±8–539±16 pg/ml; this was reversed by preincubation with calphostin C, followed by HDL (361±8 pg/ml, $n = 7$, $P < 0.05$). Calphostin C alone, at the concentration used, had little effect by itself (355±6 pg/ml). Additionally, we downregulated cytosolic PKC by incubating the cells with a phorbol ester (PMA, 1 µM) for 24 h. The secretion from control cells incubated in the presence of PMA was not significantly different from the secretion from cells incubated in the absence of this phorbol ester (352±8 vs 380±13 pg/ml). PMA preincubation almost completely reversed the HDL-stimulated ET-1 secretion (539±16 vs 388±16 pg/ml, $n = 7$, $P < 0.05$). These studies indicate that the ability of HDL to stimulate ET-1 secretion is dependent on PKC activation. This mechanism of HDL's action is presumably related to the phosphorylation by PKC of key substrate proteins necessary for ET-1 production (translation).

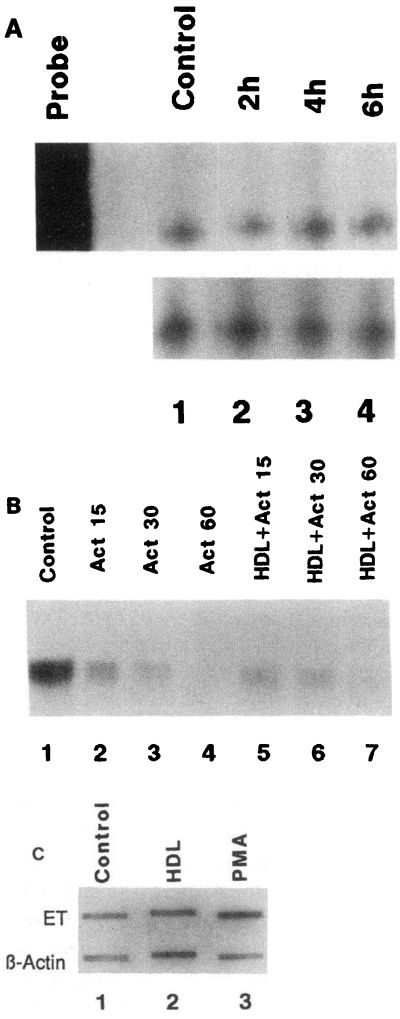

Figure 3. (*A*) Steady state ET-1 mRNA levels from cells incubated with HDL, determined by S1 nuclease protection. (*Upper panel*) Lanes *1–4* are the signal from the hybridization of 20 mg of total RNA extracted from control BAEC (no HDL) and cells incubated with HDL 50 mg/ml for 2, 4, and 6 h, respectively. The two lanes to the left of lane *1* are the labeled cRNA probe alone and the probe digested with S1 nuclease in the absence of RNA. The lower panel is the same amount of RNA from each experimental condition, hybridized with a cRNA probe for H-*ras* as an RNA loading control. The figure shown is representative of three separate experiments. (*B*) ET-1 transcript half-life studies. BAEC were incubated in the presence of actinomycin D 10^{-6} M or actinomycin D plus HDL 50 mg/ml, for 15, 30, or 60 min. The cells were then lysed, and the total RNA was extracted and hybridized with a cRNA probe for ET-1 as described. The calculated ET-1 mRNA half-life is ~ 15 min and was unaffected by HDL, based upon combined results from three studies. (*C*) Nuclear run-on study of the effects of HDL 50 mg/ml or phorbol ester (*PMA*) 10^{-7} M on ET-1 transcription after 4 h of incubation with the BAEC. β-actin transcription was also determined for comparison. This experiment was repeated two additional times; densitometry values in the text are derived from the three experiments combined.

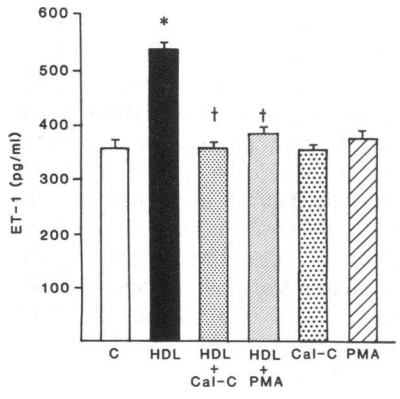

Figure 4. The effects of PKC on HDL-stimulated ET-1 secretion from BAEC after 4 h of incubation. Cells were preincubated with calphostin C, 50 nM, for 30 min before HDL, 10 μg/ml, or with PMA, 1 μM, for 24 h before HDL addition. Results are mean±SEM values from two experiments combined (n = 7 per experimental condition), representative of a third study. $^*P < 0.05$ by ANOVA plus Scheffe's test for control vs HDL, $^\dagger P < 0.05$ for HDL vs HDL plus calphostin C, or HDL plus PMA.

Discussion

Our findings indicate that HDL stimulates the production and secretion of ET-1 from cultured BAEC, as a result of stimulating the translation of this protein. There was no significant effect of HDL on the ET mRNA steady state levels, new transcription, or transcript degradation in these cells. The actions of HDL occur at low concentrations and therefore could be relevant to the in vivo action of this lipoprotein. It has been shown, for instance, that HDL binds cells with high affinity to promote cholesterol efflux and that these actions saturate at HDL protein concentrations of ~ 20 μg/ml (19, 20). This would indicate, based upon our studies, that HDL should interact with the endothelium in vivo to regulate the production of vasoactive peptides, such as ET-1. ET-1 is a potent vasoconstrictor and hence plays a role in the modulation of vasomotor tone (9). Excessive secretion and subsequent action of this peptide have been implicated in the development of both acute and chronic vascular disease, including atherosclerosis (12, 13, 21), and therefore understanding the regulation of ET-1 by other vascular factors is important.

The action of HDL is probably mediated through its predominant apoprotein, apo A-I. We found that this apoprotein potently stimulates ET-1 production and secretion ~ 85% comparably with HDL and therefore could predominantly account for the increased concentration of ET-1 caused by this lipoprotein. It has recently been shown that apo A-I binds to membranes prepared from BAEC with high affinity and capacity (22). In fact, the calculated binding K_d in these studies (44 μg/ml) is comparable with the concentrations of HDL used in our study, concentrations which significantly augment ET-1 production. Apo A-I presumably binds to a transmembrane receptor protein (which has not been isolated) and induces a second messenger signal, which leads to its actions on the endothelial cell such as stimulating the secretion of ET-1. The HDL-generated signal which is necessary to stimulate ET-1 secretion from BAEC appears to involve the activation of PKC. In our studies, both PKC downregulation or antagonism prevented this action of HDL. It has previously been shown that HDL-dependent efflux of cholesterol from cells is mediated by the stimulation of diacylglycerol production and PKC activation (23, 24). Thus, another potentially important action of HDL in the endothelium could occur through this mechanism.

Other lipoproteins might also modulate endothelin secretion. Recently, two groups have studied the action of LDL on ET-1 production and secretion from cultured BAEC. Boulanger and associates (25) found that oxidized, but not native, LDL increased the mRNA and release of ET-1 from cultured human and porcine endothelial cells or intact blood vessels, through a PKC-related mechanism. This effect probably occurred through the scavenger receptor, since acetylated LDL also stimulated ET-1, while dextran sulfate, a scavenger receptor antagonist, inhibited the LDL stimulation. In contrast, Jougasaki et al. (26) found that oxidized LDL inhibited ET-1 secretion from cultured porcine or human endothelial cells, an effect probably mediated through the lysophosphatidylcholine moiety of the oxidized LDL. Thus, it is unclear as to what are the interactive effects of LDL with ET-1 production. Overall, these studies, including ours, indicate there may be important interactions between cholesterol processing and action and ET-1 production in the vasculature.

The mechanism by which HDL stimulates ET-1 production appears to result mainly from the regulation of translation. We could find little important influence of HDL on ET-1 transcription, steady state mRNA expression, or transcript degradation, despite extensive studies. This is perhaps surprising since the ET-1 gene promoter contains a phorbol ester–responsive AP-1 site and is transcriptionally stimulated by PMA (27). We confirm the fact that phorbol ester can stimulate the transcription of ET-1 and speculate that HDL might induce a repressor or other inhibitory mechanism that interferes with the ability of PKC to transactivate the ET-1 gene. Alternatively, HDL-stimulated PKC activation in cultured endothelial cells may not be equivalent to kinase activation by phorbol esters.

The regulation of any important vasoactive peptide by another protein, primarily at the level of translation, has not been previously reported and is certainly unique for the modulation of endothelin production. In fact, it is uncommon in general for protein synthesis to be modulated primarily at the level of translation. The best characterized example of this phenomenon is the regulation of the ferritin protein by iron (28). Klausner and Harford (28) have shown that the ferritin mRNA contains a *cis*-acting element, the IRE (28). An IRE-binding protein acts at this site on the ferritin mRNA as a repressor (29) and is functionally enhanced by a lack of iron, leading to decreased ferritin translation (30, 31). The mechanism by which HDL affects ET-1 translation cannot be determined from the studies reported here. Based upon our findings, however, we speculate that the activation of PKC may lead to the phosphorylation and activation of a regulatory protein that enhances the translation of the mature, cytoplasmic ET-1 mRNA. Recently, we reported that atrial natriuretic peptide inhibits the translation and secretion of ET-1 from cultured endothelial cells and prevents the degradation of the ET-1 transcript (14). These two events are probably related and perhaps result from the inhibition of the synthesis of an enzyme (RNAse) or critical amino acid sequence necessary for transcript degradation (32, 33). The regulation of ET-1 by HDL and atrial natriuretic peptide thus provides a model to understand the events and interactions governing ET-1 translation and transcript processing.

HDL is a well recognized inhibitor of the development of atherosclerosis, and this action is attributable to its role as a cholesterol scavenger and promoter of cholesterol efflux from the cell (2–4). Our results suggest that the vascular protective nature of HDL can occur despite potentially deleterious effects mediated through ET-1 production and subsequent action, if these interactions occur chronically in the in vivo state. However, it has recently been shown that HDL which contains both apo A-I and apo A-II is much less antiatherogenic than HDL which contains only apo A-I (34). In these studies, the two subfractions were not separated.

It is certainly possible that HDL might also influence the vasoconstrictor or other actions of ET (9), apart from stimulating the production of ET. As a precedent for this type of interaction, we recently showed that insulin can strongly increase ET-1 production, secretion, and mitogenic action, in vitro and in vivo (15). The interaction of HDL with other growth factors in the vascular endothelium is well recognized. For instance, HDL strongly stimulates DNA synthesis in cultured vascular endothelial cells (5, 35), perhaps through the activation of PKC, c-*fos* and c-*myc* (36–38), and the mitogenic effect of HDL is increased by fibroblast growth factor (5). Our findings lead us to propose that it is the stimulation of ET production by HDL that might ultimately play a role in the actions of ET-1. Concerning this, we have recently found in preliminary studies that HDL is a potent but slowly acting vasoconstrictor on isolated aortic rings and that this effect is lost after denuding the endothelium. Experiments are in progress to determine whether HDL-induced stimulation of ET secretion, and hence action, underlies these effects, based upon what we report here in vitro.

In summary, we found that human HDL strongly stimulate the production of ET-1 from cultured endothelial cells. This effect of HDL uniquely results mainly from the modulation of protein translation. This probably happens via the apo A-I apoprotein and through the activation of PKC. Thus, dynamic interactions of lipoproteins and vasoactive peptides probably occur at several levels, including production. Understanding these effects in vivo should provide important insights into the function of the normal vasculature and the development of arterial disease.

Acknowledgments

This work was supported by grants from the Research Service of the Veterans Administration and National Institutes of Health grant 1RO1HL50161 (E. R. Levin).

References

1. Ross, R. 1986. The pathogenesis of atherosclerosis: an update. *N. Engl. J. Med.* 314:488–500.

2. Gordon, D., and B. M. Rifkind. 1989. High-density lipoprotein: the clinical implications of recent studies. *N. Engl. J. Med.* 321:1311–1316.

3. Glomset, J. A. 1968. The plasma lecithins: cholesterol acyltransferase reaction. *J. Lipid Res.* 9:155–167.

4. Kashyap, M. L. 1989. Basic considerations in the reversal of atherosclerosis: significance of high density lipoproteins in stimulating reverse cholesterol transport. *Am. J. Cardiol.* 63:56H–59H.

5. Tauber, J.-P., J. Cheng, and D. Gospodarowicz. 1980. Effect of high and low density lipoproteins on proliferation of cultured bovine vascular endothelial cells. *J. Clin. Invest.* 66:696–708.

6. Gospodarowicz, D., S. Massoglia, J. Cheng, and D. K. Fujii. 1986. Effect of fibroblast growth factor and lipoproteins on the proliferation of endothelial cells derived from bovine adrenal cortex, brain cortex, and corpus luteum capillaries. *J. Cell. Physiol.* 127:121–136.

7. Oppenheimer, M. J., K. Sundquist, and E. L. Bierman. 1989. Downregulation of high density lipoprotein receptor in human fibroblasts by insulin and IGF-1. *Diabetes.* 38:117–122.

8. Oppenheimer, M. J., J. F. Oram, and E. L. Bierman. 1987. Downregulation of high density lipoprotein receptor activity of cultured fibroblasts by platelet-derived growth factor. *Arteriosclerosis.* 7:325–332.

9. Yanagisawa, M., H. Kurihara, S. Kimura, Y. Tomobe, M. Kobayashi, Y. Mitsui, Y. Yazaki, K. Goto, and T. Masaki. 1988. A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature (Lond.).* 332:411–415.

10. Yanagisawa, M., and T. Masaki. 1989. Molecular biology and biochemistry of the endothelins. *Trends Pharmacol. Sci.* 10:374–378.

11. Lerman, A., F. L. Hildebrand, K. B. Margulies, B. O'Murchu, M. A. Perrella, D. M. Heublein, T. R. Schwab, and J. C. Burnett. 1990. Endothelin: a new cardiovascular regulatory peptide. *Mayo Clin. Proc.* 65:1441–1455.

12. Miyauchi, T., M. Yanagisawa, T. Tomizawa, Y. Sugishita, N. Suzuki, M. Fujino, R. Ajisaka, K. Goto, and T. Masaki. 1989. Increased plasma concentrations of endothelin-1 and big endothelin-1 in acute myocardial infarction. *Lancet.* i:53–54.

13. Dubin, D., R. E. Pratt, J. P. Cooke, and V. J. Dzau. 1989. Endothelin, a potent vasoconstrictor, is a vascular smooth muscle mitogen. *J. Vasc. Med. Biol.* 1:150–154.

14. Hu, R.-M., E. R. Levin, A. Pedram, and H. J. L. Frank. 1992. Atrial natriuretic peptide inhibits the translation and secretion of endothelin from cultured bovine aortic endothelial cells: mediation through C receptors. *J. Biol. Chem.* 267:17384–17389.

15. Hu, R.-M., E. R. Levin, A. Pedram, and H. J. L. Frank. 1993. Insulin increases the production and secretion of endothelin from cultured bovine endothelial cells. *Diabetes.* 42:351–358.

16. Svetlov, S., and S. Nigam. 1993. Calphostin C, a specific protein kinase C inhibitor, activates human neutrophils: effect on phospholipase A2 and aggregation. *Biochim. Biophys. Acta.* 1177:75–78.

17. Schumaker, V. N., and D. L. Puppione. 1986. Sequential flotation ultracentrifugation. *Methods Enzymol.* 128:155–170.

18. Bloch, K. D., S. P. Friedrich, M.-L. Lee, R. L. Eddy, T. B. Shows, and T. Quertermous. 1989. Structural organization and chromosomal assignment of the gene encoding endothelin. *J. Biol. Chem.* 264:10851–10857.

19. Oram, J. F., J. J. Albers, M. C. Cheung, and E. L. Bierman. 1981. The effects of subfractions of high density lipoprotein on cholesterol efflux from cultured fibroblasts. *J. Biol. Chem.* 256:8348–8356.

20. Oram, J. F., E. A. Brinton, and E. L. Bierman. 1983. Regulation of high density lipoprotein receptor activity in cultured human skin fibroblasts and human arterial smooth muscle cells. *J. Clin. Invest.* 72:1611–1621.

21. Watanabe, T., N. Suzuki, N. Shimamoto, M. Fujino, and A. Imada. 1990. Endothelin in myocardial infarction. *Nature (Lond.).* 344:114.

22. Vadiveloo, P. K., and N. H. Fidge. 1992. The role of apoproteins AI and AII in binding of high density lipoprotein$_3$ to membranes derived from bovine aortic endothelial cells. *Biochem. J.* 284:145–151.

23. Theret, N., C. Delbart, G. Aguie, J. C. Fruchart, G. Vassaux, and G. Ailhaud. 1990. Cholesterol efflux from adipose cells is coupled to diacylglycerol production and protein kinase C activation. *Biochem. Biophys. Res. Commun.* 173:1361–1368.

24. Mendez, A. J., J. F. Oram, and E. L. Bierman. 1991. Protein kinase C as a mediator of high density lipoprotein receptor-dependent efflux of intracellular cholesterol. *J. Biol. Chem.* 266:10104–10111.

25. Boulanger, C. M., F. C. Tanner, M.-L. Bea, A. W. A. Hahn, A. Werner, and T. F. Luscher. 1992. Oxidized low density lipoproteins induce mRNA expression and release of endothelin from human and porcine endothelium. *Circ. Res.* 70:1191–1197.

26. Jougasaki, M., K. Kiyotaka, Y. Saito, K. Nakao, H. Imura, and H. Yasue. 1992. Suppression of endothelin-1 secretion by lysophosphatidylcholine in oxidized low density lipoprotein in cultured vascular endothelial cells. *Circ. Res.* 71:614–619.

27. Inoue, A., M. Yanagisawa, Y. Takuwa, Y. Mitsui, M. Kobayashi, and T. Masaki. 1989. The human preproendothelin gene: complete nucleotide sequence and regulation of expression. *J. Biol. Chem.* 264:14954–14959.

28. Klausner, D., and J. B. Harford. 1989. Cis-trans model for post-transcriptional gene regulation. *Science (Wash. DC).* 246:870–872.

29. Goossen, B., S. W. Caughman, J. B. Harford, R. D. Klausner, and M. W. Hentze. 1990. Translational repression by a complex between the iron-responsive element of ferritin mRNA and its specific cytoplasmic binding protein is position-dependent in vivo. *EMBO (Eur. Mol. Biol. Organ.) J.* 9:4127–4133.

30. Hentze, M. W., T. A. Roualt, J. B. Harford, and R. D. Klausner. 1989. Oxidation-reduction and the molecular mechanism of a regulatory RNA-protein interaction. *Science (Wash. DC).* 244:357–359.

31. Caughman, S. W., M. W. Hentze, T. A. Roualt, J. B. Harford, and R. D. Klausner. 1988. The iron-responsive element is the single element responsible for iron-dependent translational regulation of ferritin biosynthesis. *J. Biol. Chem.* 263:19048–19052.

32. Shaw, G., and R. Kamen. 1986. A conserved AU-rich sequence from the 3′ untranslated region of GM-CSF mediates selective mRNA degradation. *Cell.* 55:1115–1122.

33. Cleveland, D. W., and T. J. Yen. 1989. Multiple determinants of eukaryotic mRNA stability. *New Biol.* 1:121–126.

34. Schultz, J. R., J. G. Verstuyft, E. L. Gong, A. V. Nichols, and E. M. Rubin. 1993. Protein composition determines the anti-atherogenic properties of HDL in transgenic mice. *Nature (Lond.).* 365:762–764.

35. Darbon, J.-M., F.-F. Tournier, J.-P. Tauber, and F. Bayard. 1986. Possible role of protein phosphorylation in the mitogenic effect of high density lipoproteins on cultured vascular endothelial cells. *J. Biol. Chem.* 261:8002–8008.

36. Libby, P., P. Miao, J. M. Ordovas, and E. J. Schaefer. 1985. Lipoproteins increase growth of mitogen-stimulated arterial smooth muscle cells. *J. Cell. Physiol.* 124:1–8.

37. Hahn, A. W. A., F. Ferracin, F. R. Buhler, and A. Pletscher. 1991. Modulation of gene expression by high and low density lipoproteins in human vascular smooth muscle cells. *Biochem. Biophys. Res. Commun.* 178:1465–1471.

38. Komuro, I., H. Kurihara, T. Sugiyama, F. Takaku, and Y. Yazaki. 1988. Endothelin stimulates c-fos and c-myc expression and proliferation of vascular smooth muscle cells. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 238:249–252.