Endothelin-1 decreases CD36 protein expression in vascular smooth muscle cells

Ching Fai Kwok,1,3 Chi-Chang Juan,2,4 and Low-Tone Ho1,2,3,4

1Division of Endocrinology and Metabolism, Department of Medicine and 2Department of Medical Research & Education, Taipei Veterans General Hospital; and 3Faculty of Medicine and 4Institutes of Physiology and Clinical Medicine School of Medicine, National Yang-Ming University, Taipei, Taiwan.

ET-1 DECREASES CD36 EXPRESSION IN VSMCS

This work was supported by grant from Taipei Veterans General Hospital (VGHUST93-P1-03, VGHUST 94-P1-11 and VGH94-219).

Address for reprint requests and other correspondence: Ching Fai Kwok, M.D., Division of Endocrinology and Metabolism, Taipei Veterans General Hospital, Taipei, Taiwan.

(E-mail: cfkwok@vghtpe.gov.tw; Fax: 886-2-2872-4982)

Keywords: endothelin-1, CD36, smooth muscle cell, atherosclerosis.
ABSTRACT

Recent studies have shown that CD36 plays important roles as a major scavenger receptor for oxidized low-density lipoproteins and as a crucial transporter for long-chain fatty acids. CD36 deficiency might be associated with insulin resistance and abnormal dynamics of long-chain fatty acids. Endothelin-1 (ET-1), which is synthesized and secreted by vascular endothelial cells, is the most potent endogenous vasoconstrictor known and also stimulates the proliferation of vascular smooth muscle cells (VSMCs) and thus is believed to play an important role in the development of various circulatory disorders, including hypertension and atherosclerosis. The aim of the present study was to investigate the regulatory effect of ET-1 on CD36 expression in cultured VSMCs. VSMCs were treated for different times (0-24 hours) with a fixed concentration (100 nM) of ET-1 or with different concentrations (0-100 nM) for a fixed time (24 h), then CD36 expression was determined using Western blots. CD36 expression was significantly decreased by ET in a time- and dose-dependent manner. This inhibitory effect was prevented by the ET_{A} receptor antagonist, BQ-610 (10 µM), but not the ET_{B} receptor antagonist, BQ-788 (10 µM). To further explore the underlying mechanisms of ET-1 action, we examined the involvement of the tyrosine kinase-mediated and mitogen-activated protein kinase-mediated pathways. The inhibitory effect of ET-1 on CD36 protein expression was blocked by inhibition of tyrosine kinase activation using genistein (100 µM) and by the ERK inhibitor, PD98059 (75 µM), but not the p38MAPK inhibitor, SB203580 (20 µM). In conclusion, we have demonstrated that ET-1, acting via the ET_{A} receptor, suppresses CD36 protein expression in VSMCs by activation of the tyrosine kinase and ERK pathways.
INTRODUCTION

Atherosclerosis and coronary heart disease are the leading causes of mortality and morbidity in the developed world. During the atherosclerotic process, lipoproteins, such as low density lipoprotein (LDL), are converted to oxidized-LDL (ox-LDL), which contains modified proteins and lipids (9). A family of membrane proteins, the scavenger receptors, recognizes and internalizes modified lipoproteins, making them susceptible to degradation. However, uncontrolled expression of scavenger receptors can lead to foam cell formation, one of the major histopathological features of atherosclerosis (30). Although each scavenger receptor shows a broad specificity, clear preferences for certain ligands have been described (37). The CD36 scavenger receptor is specific for nitrated LDL and ox-LDL, the most atherogenic forms of modified LDL (31). CD36, originally identified as glycoprotein IV on platelets, is an 88 kDa integral membrane protein that has multiple ligands and it is widely expressed in human tissues, including heart, skeletal muscles, adipose tissues, blood vessel walls, and intestines (2,26,42). CD36 expression is increased in macrophages in human atherosclerotic lesions (17,30). Moreover, CD36 knockout mice exhibit a reduced uptake of modified LDL and a reduction in the occurrence of atherosclerosis (14). These results indicate that macrophage CD36 plays an important role in the development of atherosclerosis. However, the role of CD36 in vascular smooth muscle cell (VSMC) in the pathogenesis of atherosclerosis is not as clear as that of macrophage. Finally, CD36 deficiency has been shown to be associated with insulin resistance (28).

Recent studies have shown that CD36 is also a crucial transporter for long-chain fatty acids (LCFAs). Unlike sugars, amino acids, and nucleotides, LCFAs are apolar compounds and readily partition into the membrane, which adds complexity to the transport of LCFAs across the cellular membrane. However, cell studies have provided evidence for the involvement of high-affinity protein components in LCFA transport in adipose tissue (3,4),
liver (36,40), skeletal muscle and heart (27). This led to the identification of three major potential fatty acid (FA) transporting proteins, plasma membrane FA binding protein (FABPPM), CD36 (FA translocase), and FA transport protein (FATP) (1,18,34,38). Although evidence supporting a role for FATP and for FABPPM in FA metabolism has been obtained, CD36 plays the pivotal role.

CD36 is a tightly regulated protein. Known regulatory mechanisms are at the level of mRNA, mediated by PPARs (41), and at the translational level, mediated by glucose (16). Moreover, acute regulation of FA transport by insulin is accomplished at the protein level by translocation of CD36 from an intracellular store to the plasma membrane (25). All three regulatory mechanisms appear to be relevant to the development of diseases and their associated complications.

Endothelin-1 (ET-1), synthesized and secreted by vascular endothelial cells, is by far the most potent endogenous vasoconstrictor known (43). It also stimulates DNA synthesis in vascular smooth muscle cells (VSMCs) and thus is suspected to play an important role in the development of various circulatory disorders, including hypertension and atherosclerosis (29). After being secreted from endothelial cells, ET-1 binds to specific receptors on nearby VSMCs and perhaps on VSMCs at other distal sites (35). Two distinct types of ET receptors, ET\textsubscript{A} and ET\textsubscript{B}, have been cloned and sequenced from bovine and rat tissues (8,33). ET\textsubscript{A} receptors predominate in VSMCs and mediate vasoconstriction and cellular proliferation (13,19). Recently, we demonstrated overexpression of vascular ET\textsubscript{A} receptors in a fructose-induced hypertensive rat model, which further supports the role of ET-1 and its receptors in hypertension (22).

The aim of the present study was to investigate the effect of ET-1 on CD36 expression and its underlying regulatory mechanisms. Since both CD36 and ET-1 are involved in the pathology of atherosclerosis, this was expected to provide valuable information about the
regulation of CD36 expression and to contribute to a better understanding of the development of atherosclerosis.

MATERIALS AND METHODS

Materials. ET-1 was purchased from the Peptide Institute, Osaka, Japan, BQ-610 and BQ-788 from Phoenix Pharmaceuticals, Belmont, CA, and genistein, PD098059, and SB203580 from BIOMOL International, Plymouth Meeting, PA.

Experimental Design. To explore the regulatory effect of ET-1 on CD36 protein expression, VSMCs were treated for different times (0-24 hours) with a fixed concentration (100 nM) of ET-1 or with different concentrations (0-100 nM) for a fixed time (24 h), then CD36 protein was measured using Western blots. To identify the receptor subtype(s) involved in the ET-1-mediated inhibition of CD36 protein expression, VSMCs were incubated for 1 h in the presence or absence of 10 μM BQ-610 (an ET_A receptor antagonist) or 10 μM BQ-788 (an ET_B receptor antagonist), then 100 nM ET-1 was added, and the cells incubated for a further 24 h. To explore the underlying mechanisms of ET-1 action, VSMCs were preincubated for 1 h in the presence or absence of a tyrosine kinase inhibitor (genistein; 100 μM), an ERK inhibitor (PD98059; 75 μM), or a p38 MAP kinase inhibitor (SB203580; 20 μM), then with 100 nM ET-1 in the continued presence or absence of these inhibitors for a further 24 h. The cell content of CD36 was then determined.

Culture of vascular smooth muscle cells (VSMCs). This was performed as described previously (24). Briefly, male Sprague Dawley rats (150-200 g body weight) were decapitated, the thoracic aortas sterilely dissected out, and the adventitia and intima
completely removed. The medial layers were then sliced into 2×2 mm squares, which were transferred to gelatin-coated 60 mm diameter tissue culture dishes, and incubated in DME low glucose medium containing 100 units/ml of penicillin and 100 µg/ml of streptomycin (all from Gibco BRL, Gaithersburg, MD), and 10% fetal bovine serum (Biowest, Nuaillé, France) in a 37°C humidified incubator with an atmosphere of 95% air and 5% CO₂. Between days 5 and 7 after explant isolation, VSMCs started to migrate out of the explants and proliferate. At day 14, the explants were removed using fine forceps and the cells trypsinized and subcultured at a ratio of 1:3 into other dishes. Confluent VSMC cultures showed the characteristic “hill and valley” growth pattern and were able to form multilayers in culture. The cells were then harvested by treatment with 0.05% trypsin in 0.2% EDTA in serum-free medium and seeded in gelatin-coated tissue culture dishes. Cells from passages 5 to 15 were used in these studies. Before each experiment, cells were incubated for 8 h in the absence of serum, using DME low glucose medium.

**Western Blots.** After treatment, the cells were washed twice with PBS, then lysed with 0.5 mL of lysis buffer (1% Triton X-100, 150 mM NaCl, 10 mM Tris pH 7.4, 1 mM EDTA, 1 mM EGTA, 0.2 mM PMSF, 0.5% Nonidet P-40). Protein concentrations of lysates were measured using a Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA), then 100 µg of lysate proteins was separated by SDS-PAGE using a 7.5% polyacrylamide gel and electroblotted onto a PVDF membrane. After blocking of nonspecific binding by incubation for 1 h with 5% nonfat milk in PBS containing 0.05% Tween 20 (TPBS), the membrane was incubated for 1 h at room temperature with polyclonal antibodies against CD36 or α-tubulin (both from Santa Cruz, CA), washed four times with TPBS, incubated for 1 h at room temperature with horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (Santa Cruz, CA), then washed four times with TPBS. To measure ERK phosphorylation,
antibodies against phosphorylated or total ERK 1/2 (Santa Cruz, CA) were used. Bound antibody was visualized using a Western Blot Chemiluminescence Reagent Plus Kit and the intensity of the bands quantified using a densitometer (Molecular Dynamics, Sunnyvale, CA).

Statistical analysis. Experiments were repeated at least three times. The results are expressed as the mean ± SD. Statistical significance was assessed by one way analysis of variance or Student’s t test, a value of P<0.05 being considered statistically significant.

RESULTS

ET-1 decreases CD36 protein expression in VSMCs. As shown in Fig. 1, a significant decrease (about 20%) in CD36 expression was observed after 6 h of incubation with ET-1, maximal inhibition (about 50%) being seen at 12 h and maintained for at least 24 h. To examine if the effect was dose-dependent, VSMCs were incubated for 24 h with various concentrations of ET-1 (0-100 nM) and the results showed that 1 nM ET-1 induced significant inhibition of CD36 protein expression (about 30% inhibition compared to the control group), maximal inhibition of approximately 50% being seen at 10 nM (Fig. 2).

Effect of ET receptor antagonists on the ET-1-mediated inhibition of CD36 protein expression. To examine the contribution of the two ET receptors to the ET-1-mediated inhibition of CD36 expression, VSMCs were pretreated for 1 h with the ETₐ receptor antagonist BQ-610 (10 µM) or the ETₐ receptor antagonist BQ-788 (10 µM), then 100 nM ET-1 was added, and the cells incubated for 24 h. As shown in Fig. 3, BQ-610 or BQ-788 alone had no effect on CD36 protein expression, but BQ 610 completely prevented the inhibitory effect of ET-1 on CD36 expression, while BQ-788 had no effect. These results
demonstrate that the inhibition of CD36 expression by ET-1 in VSMCs is mediated through the ETA receptor.

**Effects of a tyrosine kinase inhibitor and MAP kinase inhibitors on the ET-1-mediated inhibition of CD36 protein expression.** In order to further explore the signaling mechanism by which ET-1 suppressed CD36 protein expression, VSMCs were pretreated with inhibitors of tyrosine kinase, ERK, or p38MAP kinase, and their effect on ET-1-mediated inhibition of CD36 expression evaluated. As shown in Fig. 4, pretreatment with a tyrosine kinase inhibitor (genistein; 100 µM) or an ERK inhibitor (PD98059, 75 µM), but not a p38MAP kinase inhibitor (SB203580; 20 µM), completely blocked the effect. These finding indicate that activation of tyrosine kinase and ERK, but not p38MAP kinase, is necessary for the suppressive effect of ET-1 on CD36 protein expression in VSMCs.

**Effects of ET-1 on ERK phosphorylation.** Western blots were used to determine whether ET-1 induced ERK phosphorylation. VSMCs were treated without (0 min) or with 100 nM ET-1 for 5-60 min, then ERK phosphorylation was measured by immunostaining with antibodies against phosphorylated or total ERK 1/2 (Santa Cruz, CA) which detect the phosphorylation status of ERK 1/2 (p42 and p44). The results showed that ET-1 caused a time-dependent increase in ERK phosphorylation, which peaked at 5 min, decreased at 15 min, and was no longer seen at 30 min (data not show).

**DISCUSSION**

In this study, we used primary rat aortic VSMCs as an *in vitro* model to examine the role of ET-1 in the regulation of CD36 protein expression. We discovered that, as in human aortic SMCs (32), CD36 scavenger receptor is also expressed in rat VSMCs. Our results
also demonstrated that ET-1 caused time- and dose-dependent downregulation of CD36 protein expression in VSMCs via the ET\textsubscript{A} receptor. This suppressive effect of ET-1 involved the activation of tyrosine kinase and ERK. To the best of our knowledge, this is the first indication of regulation of CD36 by ET-1 in VSMCs. Recently, Amiri et al established a new murine model with endothelium-restricted overexpression of human ET-1. In this model, human ET-1 induced vascular remodeling and endothelial dysfunction in the absence of significant increases in blood pressure (7). It will be very interesting to investigate the expression of VSMC CD36 in this animal model and to explore the role of VSMC CD36 in vascular remodeling and endothelial dysfunction.

Studies on a possible role of ET-1 in lipid metabolism are very limited. Our previous study demonstrated that ET-1 affects lipid metabolism by stimulating adipocyte lipolysis (21). In the present study, we showed that ET-1 might also manipulate homeostasis of lipid metabolism by downregulating CD36 expression in VSMCs. Since VSMCs play a pivotal role in the maintenance of vascular function, ET-1-induced CD36 downregulation might alter the properties of VSMCs and thus contribute to the development of several vascular diseases, such as hypertension and atherosclerosis. CD36 has been identified as an FA receptor/transporter (1). CD36 deficiency might result in defective clearance of FAs from the circulation and elevated blood FA levels and secondary hypertriglyceridemia (6). On the other hand, as CD36 functions as a receptor for ox-LDL and other lipoproteins (31), CD36 deficiency may reduce lipoprotein clearance. This notion is supported by Aitman’s study (5), which demonstrated that CD36-deficient spontaneously hypertensive rats have defective FA and glucose metabolism, leading to dysregulation of cardiovascular functions, insulin resistance, and diabetes.

Our study also demonstrated the ET-1-inhibited CD36 expression was mediated through ERK-dependent but not p38 MAPK-dependent pathway (Fig. 4). This signal transduction
mechanism is compatible with Bisotto’s finding (10) that ET-1 is able to stimulate Src family tyrosine kinases, which may mediate ERK activation. In addition, Chen’s study demonstrated ET-1 stimulated VSMC proliferation through two complementary signal transduction cascades including ERK and p38 MAPK (12). Furthermore, the ET<sub>A</sub> receptor-mediated growth-promoting effect of ET-1 requires activation of ERK via transactivation of epidermal growth factor (EGF) in rat VSMCs (20). Further studies are needed to clarify the role of EGF transactivation in the ET-1-mediated downregulation of CD36 expression in VSMCs.

The relevance of CD36 downregulation to pathobiology with specific respect to vascular smooth muscle cells is not clear. Deficiency of CD36 caused a significant increase in fasting levels of plasma triglycerides, cholesterol and free fatty acids (5,14). Patients with CD36 deficiency had increased plasma triglyceride and glucose, lower plasma HDL-cholesterol, and hypertension than did controls (28). Furthermore, the postprandial hyperlipidemia also occurred in patients with this monogenic disorder (23). These observations suggested that CD36 deficiency may provide a more atherogenic environment to accelerate the development of atherosclerosis. Whether this hypothesis is correct and operative in human beings must await the findings of further studies.

In the present study, we used genistein to prove that ET-1 decreased CD36 via tyrosine kinase-dependent pathway. However, the divergent effects of genistein need to be concerned. For example, it has been reported that genistein has estrogenic effects (15) and is able to inhibit cAMP-specific phosphodiesterases (11,39). These actions of genistein may confound interpretation of our results. More specific inhibitors are needed to exactly clarify the role of tyrosine kinase-dependent cascade in ET-1-decreased CD36 expression in VSMCs.

In summary, our findings indicate that ET-1, acting through the ET<sub>A</sub> receptor, decreases
CD36 protein expression in VSMCs. The underlying mechanism involves the activation of the tyrosine kinase and ERK pathways. Further characterization of CD36 regulation in response to proatherogenic and antiatherogenic stimuli could lead to the development of therapeutic strategies to prevent or reverse the progression of atherosclerosis.

ACKNOWLEDGMENT

We would like to thank Ms Ren Yeu Kwok for her excellent editorial assistance.
REFERENCES


36. **Sorrentino D, Robinson RB, Kiang CL, and Berk PD.** At physiologic albumin/oleate concentrations oleate uptake by isolated hepatocytes, cardiac myocytes, and adipocytes is a saturable function of the unbound oleate concentration. Uptake kinetics are consistent with the conventional theory. *J Clin Invest* 84: 1325-1333, 1989.


42. **Van Nieuwenhoven FA, Verstijnen CP, Abumrad NA, Willemsen PH, Van Eys GJ, Van der Vusse GJ, and Glatz JF.** Putative membrane fatty acid translocase and
cytoplasmic fatty acid-binding protein are co-expressed in rat heart and skeletal muscles.


FIGURE LEGENDS

Fig. 1. Time-dependent effect of ET-1 on CD36 protein expression. VSMCs were incubated in serum-free medium for 8 h, then in the presence or absence of 100 nM ET-1 for various times (0-24 h), and the CD36 protein content measured by Western blotting. The results are the means ± SD of three separate experiments. *P<0.05 compared to the time-zero control.

Fig. 2. Dose-dependent effect of ET-1 on CD36 protein expression. VSMCs were incubated in serum-free medium for 8 h, then with various concentrations of ET-1 (0-100 nM) for 24 h, and the CD36 protein content was measured. The results are the means ± SD of three separate experiments. *P<0.05 compared to the vehicle control.

Fig. 3. Effect of ET₄R and ET₅R antagonists on the ET-1-mediated inhibition of CD36 expression. VSMCs were incubated in serum-free medium for 8 h, preincubated for 1 h in the presence or absence of the ET₄R antagonist, BQ-610 (10 µM), or the ET₅R antagonist, BQ-788 (10 µM), then incubated in the presence or absence of ET-1 (100 nM) in the continued presence or absence of the antagonist for a further 24 h before CD36 protein content was measured. The results are the means ± SD of three separate experiments. *P<0.05 compared to the vehicle control.

Fig. 4. Effect of tyrosine kinase and MAP kinase inhibitors on the ET-1-mediated inhibition of CD36 expression. VSMCs were incubated in serum-free medium for 8 h, preincubated for 1 h in the presence or absence of the tyrosine kinase inhibitor, genistein (G; 100 µM), the ERK inhibitor, PD98059 (PD; 75 µM), or the p38MAPK inhibitor, SB203580 (SB; 20 µM), then incubated in the presence or absence of ET-1 (100 nM) in the continued presence of the inhibitor for a further 24 h before the CD36 protein content was measured. The results are the means ± SD of three separate experiments. *P<0.05 compared to the vehicle control.
Fig. 1. Time-dependent effect of ET-1 on CD36 protein expression. VSMCs were incubated in serum-free medium for 8 h, then in the presence or absence of 100 nM ET-1 for various times (0-24 h), and the CD36 protein content measured by Western blotting. The results are the means ± SD of three separate experiments. *P<0.05 compared to the time-zero control.
Fig. 2. Dose-dependent effect of ET-1 on CD36 protein expression. VSMCs were incubated in serum-free medium for 8 h, then with various concentrations of ET-1 (0–100 nM) for 24 h, and the CD36 protein content was measured. The results are the means ± SD of three separate experiments. *P<0.05 compared to the vehicle control.
Fig. 3. Effect of ETₐR and ETₐR antagonists on the ET-1-mediated inhibition of CD36 expression. VSMCs were incubated in serum-free medium for 8 h, preincubated for 1 h in the presence or absence of the ETₐR antagonist, BQ-610 (10 μM), or the ETₐR antagonist, BQ-788 (10 μM), then incubated in the presence or absence of ET-1 (100 nM) in the continued presence or absence of the antagonist for a further 24 h before CD36 protein content was measured. The results are the means ± SD of three separate experiments. *P<0.05 compared to the vehicle control.
Fig. 4. Effect of tyrosine kinase and MAP kinase inhibitors on the ET-1-mediated inhibition of CD36 expression. VSMCs were incubated in serum-free medium for 8 h, preincubated for 1 h in the presence or absence of the tyrosine kinase inhibitor, genistein (G; 100 μM), the ERK inhibitor, PD98059 (PD; 75 μM), or the p38MAPK inhibitor, SB203580 (SB; 20 μM), then incubated in the presence or absence of ET-1 (100 nM) in the continued presence of the inhibitor for a further 24 h before the CD36 protein content was measured. The results are the means ± SD of three separate experiments. *P<0.05 compared to the vehicle control.