Leptin Induces C-Reactive Protein Expression in Vascular Endothelial Cells

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Objective—There is increasing evidence of an association between leptin and increased cardiovascular risk. Higher leptin levels are associated with increased levels of C-reactive protein (CRP), which itself elicits proatherogenic effects in the vascular endothelium. We tested the hypothesis that leptin induces CRP expression in human coronary artery endothelial cells (HCAECs).

Methods and Results—We confirmed the presence of both long and short isoforms of the leptin receptor in cultured HCAECs. Leptin but not IFN-αA/D nor tumor necrosis factor (TNF) α, induced expression of CRP. A dose dependent increase of CRP mRNA and protein was observed with increasing concentration of leptin (0 to 400 ng/mL). This increased CRP expression was attenuated in the presence of anti-leptin receptor antibodies and also by inhibition of ERK1/2 by PD98059 (20 to 40 μmol/L). Time (0 to 60 minutes) and leptin concentration (0 to 200 ng/mL)-dependence of ERK1/2 phosphorylation were evident in response to leptin treatment. Leptin also elicited ROS generation. Inhibition of ROS by catalase (200 μg/mL) prevented ERK1/2 phosphorylation and CRP mRNA transcription.

Conclusion—Leptin induces CRP expression in HCAECs via activation of the leptin receptor, increased ROS production, and phosphorylation of ERK1/2. These studies suggest a mechanism for the proatherogenic effects of leptin.

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Key Words: leptin ■ endothelium ■ C-reactive protein ■ obesity ■ atherosclerosis

Leptin, a protein encoded by the Ob gene, is produced mainly by adipocytes and is primarily involved in regulation of food intake and energy expenditure. Several studies have shown an independent interaction between high leptin and atherosclerosis,1,2 myocardial infarction, stroke, and coronary artery intima-media thickness, suggesting that high levels of leptin imply increased cardiovascular risk.3 Deter- mental effects of leptin may include sympathetic activation, pressor responses, insulin resistance, platelet activation and aggregation, inflammation, oxidative stress, and proliferation and migration of vascular smooth muscle cells.3,4 The molecular mechanisms underlying the association of leptin with poor cardiovascular outcomes are not fully elucidated.

C-reactive protein (CRP), an acute phase protein, is also an indicator of cardiovascular (CV) risk.5 Localization of CRP in atherosclerotic lesions6–8 and its role in complement activation, cell adhesion, and thrombosis make it likely an important mediator in the development and progression of the atherosclerotic lesion.9 We and others have shown positive correlations between CRP and plasma leptin in normal weight and obese subjects.10,11 Exogenous administration of leptin increases plasma CRP in normal weight and fasting obese subjects.12,13 Decreases in leptin during weight loss and fasting directly correlate with decreased CRP levels.14 Induction of CRP by leptin in vascular endothelial cells would have important implications for understanding interactions between leptin and CRP in promoting CV risk. We tested the hypothesis that leptin increases CRP expression in human coronary artery endothelial cells and sought to determine the signaling pathways involved.

Methods

Experiments were performed on human coronary artery endothelial cells (HCAECs, from Cambrex, Walkersville, MD). Cells were grown in endothelial growth media-2 (EGM-2) media supplemented with growth factors and 2% FBS. All experiments were performed at 3 to 5 passages with 70% to 80% confluence after overnight incubation in serum and growth factor-free media.15

The cells were incubated with either leptin (50 to 400 ng/mL; Sigma), recombinant interferon-αA/D (IFN-αA/D; 200 U/mL; Sigma), or tumor necrosis factor-α (TNF-α; 50 ng/mL; Promega) for 24 or 48 hours for RNA or protein analysis. Interleukin (IL)-1 (20 ng/mL) and IL-6 (20 ng/mL) (R&D systems) were used as positive induction control and buffer used to make leptin stock was used as vehicle (0 leptin) control. The role of the leptin receptor in CRP induction was determined in the presence of human anti-leptin receptor antibodies (0.01 to 0.02 dilutions, Linco Research).

The effect of inhibiting activation of p38 and ERK1/2 pathway and ROS generation on CRP induction was determined in the
presence of specific inhibitors such as SB203580 (5 to 20 μmol/L; Sigma), PD98059 (20 to 40 μmol/L; Sigma), and catalase (200 μg/mL; Sigma), respectively. All inhibitory molecules were incubated with the cells for 30 minutes before leptin (100 ng/mL) treatment. To study the signaling pathway, the cells were incubated with leptin (100 ng/mL) for varying durations (0 to 60 minutes). The effect of increasing leptin concentration (0 to 200 ng/mL; 20 minutes treatment) and ROS inhibition on activation of MAPK was also determined.

ROS generation in response to leptin treatment was determined using MitoSOX red mitochondrial superoxide indicator (Molecular Probes) for live cell imaging. The cells were washed with Hank’s balanced salt solution (HBSS, Mediatech Inc) and loaded with MitoSOX (2 μmol/L) for 20 minutes before leptin (100 ng/mL) treatment. The cells were then observed in a fluorescence microscope after 20 minutes of leptin treatment to determine the ROS levels. Catalase treatment (200 μg/mL) was administered 20 minutes before the leptin treatment when needed. ROS quantification was performed using fluorescence activated cell sorting (FACS) analysis. The cells were initially treated with versene-EDTA (Cambrex), washed with HBSS, and loaded with 2′, 7′-dichlorodihydrofluorescein (1 μmol/L, H₂DCFDA; Molecular Probes) for 10 minutes at 37°C. The cells were then treated with leptin (100 ng/mL) for 15 minutes and then analyzed by FACS.

RNA was extracted from the cells after 24 hours of leptin treatment using an RNA isolation kit (Invitrogen, Carlsbad, Calif). cDNA was synthesized using total RNA (1 μg/reaction) with high capacity cDNA Archive kit (Applied Biosystems, Foster City, Calif). CRP TaqMan probe and 18sRNA TaqMan probe (endogenous control; Applied Biosystems) were used in standard conditions (as recommended by the manufacturer) to determine the level of CRP mRNA transcription in the treated cells. Absolute values of CRP and 18sRNA transcripts were calculated using standards. The CRP value obtained from the standard curve was divided by the value for endogenous control to obtain a normalized target value ratio. Results are expressed as fold increases as compared with vehicle (0 leptin) treatment. To further verify our results we used an additional endogenous control (Hu-TBP TaqMan probe in addition to 18sRNA TaqMan probe as an endogenous control with similar findings (data not shown). Furthermore, in the presence of increasing concentrations of human anti–Ob-R antibodies, an inhibitory effect on leptin-induced CRP mRNA expression was observed (Figure 2D), indicating that the leptin-dependent CRP response was mediated via the leptin receptor.

Leptin is known to activate a number of signaling pathways including mitogen-activated protein kinases (MAPKs) in different cell types. Hence we sought to determine the role of MAPKs in regulation of leptin-induced CRP production in HCAECs. Leptin showed a time-dependent (0 to 60 minutes) activation and deactivation of ERK1/2 (Figure 3A). Maximum phosphorylation of ERK1/2 was seen at 20 minutes after leptin treatment and declined to basal levels in 60 minutes. The increase in phosphorylation of ERK1/2 after 20 minutes of leptin treatment was dose-dependent (Figure 3B). Inhibition of leptin-induced CRP mRNA expression was observed after incubation with ERK inhibitor PD98059 in increasing doses (20 to 40 μg/mL; Figure 3C), suggesting that the effects of leptin on CRP are mediated through ERK. Increased ERK1/2 phosphorylation was seen only at 50

All experiments were performed at least 3 times. The data are presented as mean±SD. Statistical significance was determined using ANOVA followed by unpaired Student t test. The level of significance was set at P<0.05.

**Results**

Incubation of HCAECs with leptin resulted in a significant increase in CRP mRNA (Figure 1). Ligands such as IFN-α A/D and TNF-α did not elicit any significant changes in CRP mRNA transcription levels. However, a nonsignificant increase in CRP mRNA was observed after incubation with IL-1β and IL-6, and a significant increase was observed when the cells were incubated with both IL-1β and IL-6 simultaneously. The presence of the leptin receptor (Ob-R) in HCAECs was confirmed by Western blot (Figure 2A). Both long and short isoforms of the receptor were observed. HCAECs incubated with increasing doses of leptin (0 to 400 ng/mL) showed increased expression of CRP mRNA (Figure 2B) and protein (Figure 2C). The increases in CRP mRNA were significant (P = 0.005) and comparable to the cytokine-induced increase in CRP expression. We further verified our results using a Human TATA binding protein (Hu-TBP) TaqMan probe in addition to 18sRNA TaqMan probe as an endogenous control with similar findings (data not shown). Furthermore, in the presence of increasing concentrations of human anti–Ob-R antibodies, an inhibitory effect on leptin-induced CRP mRNA expression was observed (Figure 2D), indicating that the leptin-dependent CRP response was mediated via the leptin receptor.
ng/mL or higher leptin concentrations, which is consistent with our finding that lower leptin concentration (20 ng/mL) did not elicit any significant increase in CRP mRNA expression as compared with 0 leptin control (data not shown).

In contrast to ERK, leptin treatment did not alter the phosphorylation levels of p38 with time (Figure 3D). Also, increasing leptin concentrations had no effect on phosphorylation of p38 (Figure 3E). Finally, inhibition of p38 by increasing concentrations of SB203580 (5 to 20 μg/mL) did not affect CRP mRNA expression (Figure 3F).

Considering that the maximum activation of ERK1/2 was observed at 20 minutes instead of 10 minutes as previously reported,16 we sought to determine whether ERK1/2 phosphorylation was secondary to ROS generation. Leptin (100 ng/mL) treatment caused an increase in reactive oxygen species as indicated by increased fluorescence in leptin-treated cells as compared with vehicle control (Figure 4A). ROS generation in response to leptin treatment was inhibited by catalase (200 μg/mL) treatment. This is also evident from our FACS analysis showing increased gated events (Figure 4B) and shift in the fluorescence peak (Figure 4C) during leptin treatment, both of which tended to reverse during catalase treatment. The leptin-treated cells in presence of catalase failed to show any increased ERK1/2 phosphorylation (Figure 4D) and CRP mRNA transcription (Figure 4E), indicating that ERK1/2 phosphorylation during leptin treatment may be secondary to ROS generation.

**Discussion**

This study has 2 important and novel findings. First, leptin induces CRP expression in vascular endothelial cells, and second, increased CRP expression is evident only at high physiological concentrations of leptin (>30 ng/mL). Our studies further suggest that leptin induces CRP expression in HCAECs via activation of the leptin receptor, increased ROS production, and phosphorylation of ERK1/2 (Figure 4F).

These data are consistent with the independent positive correlation between plasma leptin and CRP,10,11 and with increases in plasma CRP after exogenous leptin administration in healthy human subjects.12,13 It was recently shown that leptin at normal physiological levels (0.5 to 20 ng/mL) induces CRP expression in primary human hepatocyte culture.17 Leptin does not appear to induce CRP expression in primary human adipocyte culture.18 However, this latter study included only CRP measurements from cell culture supernatants and did not involve any RNA studies or intracellular CRP measurements. We have been able to demonstrate in HCAECs a leptin-dependent increase in CRP at both transcriptional and translational levels, but only at physiologically higher leptin levels. Hence there appears to be a tissuespecific and dose-dependent CRP response to leptin.

Several human studies suggest an independent association between high leptin and worse cardiovascular prognosis.1,2 These clinical results are consistent with several in vivo
studies in leptin deficient ob/ob mice, leptin receptor–deficient db/db mice, and ApoE mice, which suggest that lack of leptin protects against atherosclerosis, whereas high leptin levels are proatherogenic.3,19 Administration of leptin has been shown to enhance atherosclerotic lesion formation in wild-type and ob/ob mice despite favorable changes in body weight, lipid profiles, and insulin sensitivity.19 However, the exact mechanisms whereby leptin may promote atherosclerosis remain unclear.

Vascular endothelial cells are an important component of the atherosclerotic process.20 This is especially true for coronary artery endothelial cells where endothelial cell dysfunction, inflammation, and prothrombotic states may contribute to coronary atherosclerosis, ischemia, infarction, and death. CRP is known to localize in atherosclerotic lesion formation in wild-type and ob/ob mice despite favorable changes in body weight, lipid profiles, and insulin sensitivity.19 However, the exact mechanisms whereby leptin may promote atherosclerosis remain unclear.

Our present findings suggest that the detrimental effects of leptin on coronary vasculature may be mediated at least in part by induction of intracellular endothelial CRP expression. In several studies,1,2 with both leptin and CRP in the model, CRP lost its predictive power whereas leptin remained significantly related to cardiovascular outcome, supporting the proposition that leptin is the primary mediator of increased risk. The effect of leptin on CRP production in HCAECs only at higher leptin levels is also supportive of a proatherogenic effect in the setting of hyperleptinemia as seen in obesity.

In the present study, we suggest the pathway through which leptin upregulates CRP expression in endothelial cells, and we demonstrate the role of the leptin receptor. Our findings confirm that leptin induces ROS formation,22 which we further show as a cause of ERK1/2 activation. The role for leptin in induction of CRP in endothelial cells is unique and important. Other ligands such as IFN-α A/D and TNF-α, which are known to activate ERK1/2, failed to induce CRP mRNA transcription. A limitation to our study is that it is in vitro. In an in vivo environment, interaction with proteins such as soluble leptin receptor and CRP17

Figure 3. Signaling pathway involved in leptin-dependent CRP expression in HCAECs. A, Western blot showing increased phosphorylation of ERK1/2 in response to leptin treatment (100 ng/mL) with time. Maximum phosphorylation is seen at 20 minutes after treatment. B, Western blot showing increased ERK1/2 phosphorylation in response to increased leptin concentrations. The ERK1/2 measurements were done 20 minutes after the leptin treatment. Increased ERK1/2 phosphorylation is seen only after leptin treatment at concentrations 50 ng/mL or higher. C, Quantitative mRNA analysis showing inhibition of CRP mRNA in response to increasing concentrations of ERK1/2 inhibitor PD98059. D, Western blot showing absence of any increased phosphorylation of p38 in response to leptin treatment (100 ng/mL). E, Western blot showing absence of any increased phosphorylation of p38 in response to increasing concentrations of leptin. F, Quantitative mRNA analysis showing absence of any effect of p38 inhibitor SB203580 on CRP mRNA. The Western blots are representative of the 3 independent experiments. The quantitative RNA data are represented as mean±SD of 4 independent experiments, each in triplicate. *P<0.05, **P<0.005, as compared with 0 inhibition control.

HCAEC likely plays an important role in the development and progression of atherosclerotic lesions.
may modulate leptin regulation of CRP expression. We could also speculate that leptin, via upregulation of CRP, is involved in downregulating its own activity. However the data regarding leptin-CRP interaction and its ability to interfere with leptin signaling remain controversial and needs further investigation.23

CRP is already being targeted for drug development. CRP inhibitors are capable of significantly reducing the size of myocardial infarction in the rat model.24 Our present findings may be potentially relevant to inhibiting the proatherogenic effect of leptin, and may have direct implications for prevention and treatment of cardiovascular diseases. Leptin itself,
which causes increased expression of intracellular CRP, may hence be a good therapeutic target.

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Disclosures
P.S., M.H., R.W., and V.K.S. are working with Mayo Health Solutions regarding intellectual property related to these studies.

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