Lovastatin Reduces Nuclear Factor \( \kappa B \) Activation Induced by C-Reactive Protein in Human Vascular Endothelial Cells

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The role of C-reactive protein (CRP) in atherogenesis has been supported by more recent data. Some studies have demonstrated marked up-regulation inflammatory responses in endothelial cells subjected to CRP. The nuclear factor-\( \kappa B \) (NF-\( \kappa B \)) signal transduction is known to play a key role in the expression of these proatherogenic entities. Statins have anti-inflammatory properties independent of their cholesterol-lowering effects. Therefore, we studied the effects of CRP and lovastatin on NF-\( \kappa B \) activation in human umbilical vein endothelial cells (HUVECs). By using an electrophoretic mobility shift assays (EMSA), we found that CRP (50 \( \mu \)g/ml) increased activation of NF-\( \kappa B \) and degradation of inhibitory kappa B (I\( \kappa B \)) in HUVECs, reaching a maximal effect after the incubation with CRP for 1 h. Lovastatin (10\(-5\) mol/l) diminished NF-\( \kappa B \) activation induced by CRP. Furthermore, lovastatin may block NF-\( \kappa B \) activation by causing a stabilization of the I\( \kappa B \)-\( \alpha \) in cellular cytoplasm with western blotting analysis. Preincubation of HUVECs with pyrrolidinedithiocarbamate (PDTC, NF-\( \kappa B \) inhibitor) diminished CD40 expression induced by CRP with flow cytometry. Our results suggest that CRP increases activation of NF-\( \kappa B \) and induces CD40 expression in HUVECs partly via activation of NF-\( \kappa B \). Lovastatin, through the inhibition of NF-\( \kappa B \) activation, reduces the inflammation involved in the pathogenesis of atherosclerosis.

Key words lovastatin; C-reactive protein (CRP); nuclear factor-\( \kappa B \) (NF-\( \kappa B \)); CD40

MATERIALS AND METHODS

Reagents Dulbecco’s Modified Eagle Medium (DMEM) and fetal bovine serum (FBS) were produced by Gibco RBL (Grand Island, NY, U.S.A.). Lovastatin and pyrrolidinedithiocarbamate (PDTC) were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). T4 polynucleotide kinase was from Promega (U.S.A.). Mouse anti-human CD40, I\( \kappa B \)-\( \alpha \) and I\( \kappa B \)-\( \beta \) polyclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Recombinant human CRP was purchased from Biodesign (U.S.A.). CRP was checked by SDS–PAGE, yielding a single band when 1 \( \mu \)g was loaded on the gel. Endotoxin was removed from CRP with Detoxigel column and found to be <0.125 EU/ml by limulus assay.

Cell Culture HUVECs were isolated from fresh umbilical cords obtained at normal deliveries, which was in accordance with the ethical standards formulated in the Helsinki Declaration. The umbilical vein was filled with 20 ml of 0.1% collagenase dissolved in PBS and incubated for 15 min at 37 °C. The collagenase solution was drained from the cord and collected. The cells in these pooled solutions were recovered by centrifugation at 1000 rpm for 5 min and transferred to dishes in DMEM containing 20% FBS, penicillin (100 U/ml), streptomycin (100 mg/l), and 25 \( \mu \)g/ml endothelial cell growth supplements at 37 °C in 5% CO\(_2\). The cultured cell monolayer was identified with phase-contrast microscopy. At confluency, cells were trypsinized, counted and seeded. After the cells grew for 24 h, HUVECs were further cultured in DMEM with free FBS for the experimental treatment. In all the experiments, HUVECs were used at second or third passage.

Protein Extraction and Electrophoretic Mobility Shift

Inflammation plays a critical role in atherogenesis.3) C-reactive protein (CRP) is a prototypic marker of inflammation, and has been shown in numerous prospective studies to predict cardiovascular events.2,3) CRP is both a risk marker and has been shown in numerous prospective studies to precursor to the formation of plaque and is a key mediator of this process.4—7) However, CD40-CD40L system is also shown to increase expression and activity of eNOS and release of endothelin-1 and plasminogen activator inhibitor-1, and de-
Assays (EMSA) The cells were incubated with CRP (50 μg/ml) for 0.5, 1, 2 h. In control group, DMEM with free FBS was used instead of CRP. Nuclear protein was extracted from the cells by following the method described by Schreiber et al. Protein concentration was determined by Lowry method. The levels of NF-κB in nuclear extract were analyzed by EMSA. Five micrograms of the nuclear extract was mixed with the incubation buffer, and the mixture was preincubated at 4°C for 15 min. The sequence of oligonucleotide probe (labeled with T4 kinase and [γ-32P] ATP and purified using Pharmacia Nick columns) was as follows: NF-κB consensus: 5′-GCCC ATG GGC CGA TCC CCG AAG TCC-3′. The labeled oligonucleotide was added and the mixture was incubated at room temperature for 20 min. Samples were then applied to wells with 6% non-denaturing polyacrylamide gel. The gel was dried under vacuum and exposed to X-ray film. Densitometry was performed using Bio-Rad molecular analytic software.

Western Blot Analysis Cells were incubated with CRP (50 μg/ml) for 0.5, 1, 2 h. In control group, DMEM with free FBS was used instead of CRP. The protein levels of 1xB-α and 1xB-β were determined by Western blot. A total of 20 μg of cytoplasmic proteins was separated on a 15% SDS-PAGE and transferred to a PVDF membrane. The membrane was blocked in washing solution with 5% non-fat dried milk for 30 min at 37°C. The membrane was incubated first with 1 μg/ml of primary antibody overnight at 4°C and then with a peroxidase-conjugated secondary antibody for 30 min at 37°C. The bands were detected by NBT/BCIP colorimetry.

Flow Cytometry After the cells grew for 24 h in free FBS, PDTC in DMEM was added to the wells to incubate further for 2 h and cells were then incubated with CRP (50 μg/ml) for another 24 h. In control group, DMEM with free FBS was used instead of CRP. Expression of CD40 was assessed under the above condition by flow cytometry. HUVECs (1×10⁶/ml) harvested by trypsinization in the above condition were incubated with 10 μl (200 μg/ml) of the mouse anti-human CD40 monoclon antibody for 1 h at 4°C. Subsequently, the cells were washed twice with PBS, centrifuged at 1000 rpm for 3 min before incubated (1 h, 4°C) with the goat anti-mouse FITC-conjugated IgG 30 μl (1.4 μg/ml). Finally, the cells were washed with PBS, fixed with 4% paraformaldehyde PBS and analyzed in a Becton Dickinson FACS flow cytometer using CellQuest software (Becton Dickinson). At least 5000 viable cells per condition were analyzed.

Statistical Analysis Data were presented as mean±standard deviation (S.D.), and groups were compared using ANOVA. A p value of ≤0.05 was considered significant.

RESULTS

Activation of NF-κB by CRP in HUVECs To investigate whether CRP delivers signals that activate NF-κB, nuclear factor activity was measured by EMSA using NF-κB oligonucleotide as a probe. HUVECs were stimulated with CRP (50 μg/ml) for 0.5, 1 h and 2h, and nuclear extracts were assayed for NF-κB activity. The unstimulated HUVECs with CRP in control group showed lower activation of NF-κB. However, the stimulated HUVECs with CRP significantly increased activation of NF-κB, reaching 1.5±0.5 fold (p<0.05) in 0.5 h, 2.3±0.4 fold (p<0.01) in 1 h and 1.8±0.5 fold in 2 h (Fig. 1A). Competitive assay of NF-κB indicated that the overdose unlabeled NF-κB probe competitively inhibited binding of NF-κB to the labeled probe, showing the complete disappearance of the lane, and the mutated NF-κB probe did not prevent the binding of NF-κB to the labeled NF-κB probe, showing no change of the lane (Fig. 1B). It was suggested that CRP increased activation of NF-κB in HUVECs with a maximal effect after the incubation for 1 h.

Effect of Lovastatin on the Activation of NF-κB by CRP in HUVECs The effect of lovastatin on NF-κB activation induced by CRP was investigated in HUVECs on the basis of the above result. Activity of NF-κB was measured by the same EMSA. After the preincubation of cells with lovastatin (10⁻³ mol/l) for 24 h and then using CRP 50 μg/ml as a stimulus for another 0.5 h and 1h, the results revealed that preincubation of the cells with lovastatin diminished NF-κB activation induced by CRP (p<0.01) with a maximal inhibitory effect at 1 h (p<0.01) (Fig. 2). It was suggested that lovastatin diminished NF-κB activation induced by CRP in the used concentration.

Level of 1xB Induced by CRP and Effect of Lovastatin in HUVECs To investigate the correlation of NF-κB acti-
activation with changes of the expression of IκB, levels of IκB-α and IκB-β were determined by Western blot analysis. The expression of IκB-α was revealed after the stimulation with CRP (50 μg/ml) for 0.5 h, mostly disappeared after the same stimulation for 1 h (p<0.01) and mainly recovered after the same stimulation for 2 h. The changes of expression of IκB-α and NF-κB activity with time coincided (Fig. 3A). CRP showed no significant effect on IκB-β level (Fig. 3B). The result demonstrated that CRP activated NF-κB via promoting degradation of IκB-α. After the cells were pretreated with the lovastatin (10^{-5} mol/l) for 24 h and incubated with CRP (50 μg/ml) for 1 h, there was not any change in IκB-α levels (Fig. 3C), suggesting that lovastatin could block NF-κB activation by the stabilization of IκB-α in cellular cytoplasm.

**Effects of PDTC on Expression of CD40 Induced by CRP in HUVECs** To examine whether activation of NF-κB has been linked to the expression of CD40 induced by CRP, effect of PDTC, NF-κB inhibitor, on expression of CD40 induced by CRP was observed in HUVECs with flow cytometry. CD40 expression showed a lower level in the unstimulated cells and a higher level in the stimulated cells by CRP (50 μg/ml) for 24 h in HUVECs. After the preincubation of cells with PDTC (100 μmol/l) for 2 h and then with CRP (50 μg/ml) as a stimulus for another 24 h, effect of PDTC, NF-κB inhibitor, expression of CD40 induced by CRP was explored by flow cytometry. The columns indicate the mean value of 4 times of experiments. The bars are the S.D. and asterisks indicate a significant difference (∗∗ p<0.01) between CRP and drug-treated groups.
DISCUSSION

At present, the “new view” supports the concept that vascular inflammation is the key of atherosclerotic lesion formation, progression, and eventual rupture. People have fueled exponentially increasing interest in evaluating inflammatory markers of atherosclerosis, of which high-sensitivity CRP has emerged as one of the most important. Indeed, recent studies suggest that CRP is not only an inflammatory marker of atherosclerosis and coronary events but also a mediator of these diseases, because it contributes to the substrate underlying lesion formation, plaque rupture, and coronary thrombosis through interaction with and alteration of the vascular phenotype.

NF-κB has been implicated as a key mediator of atherosclerosis. This transcription factor is a DNA binding protein complex that is usually present in the cytosol as an inactive complex. IκB, an associated protein, renders this complex inactive by shielding the nuclear localization signal. Although several inhibitor proteins have been identified (IκB-α, IκB-β, IκB-γ, and p105), IκB-α is the best characterized form of IκB. On IκB phosphorylation and its subsequent degradation, the heterodimeric NF-κB complex translocates from the cytoplasm to the nucleus, where it binds to specific DNA sequences in the promoter region of several genes and up-regulates their transcription. Most proinflammatory genes expressed in endothelial cells during the initial phase of lesion formation and in response to inflammatory mediators are dependent on NF-κB activation. Because CRP up-regulates the production of a number of endothelial adhesion molecules known to be transcriptionally activated by NF-κB, our previous studies found that CRP, at concentration known to predict adverse cardiovascular events, caused a marked and dose-dependent, time-dependent increase in the cell-surface expression of CD40 and CD40L protein in HUVECs. But it is not know which transcription factors regulate CD40 expression. Our present studies were to assess whether CRP delivers signals that induce activation of transcription factor NF-κB and CRP induces CD40 expression via partly activation of NF-κB in HUVECs involved in atherosclerosis. The studies demonstrated that CRP increased the activation of NF-κB and the degradation of IκB-α, but not IκB-β in HUVECs, reaching a maximal effect after the incubation for 1 h. NF-κB is found in the cytoplasm in binding way to 1xIκB in the unstimulated cells, which prevents it from entering the nucleus. When these cells are stimulated with CRP, the specific kinases phosphorylate IκB to cause its rapid degradation by proteasomes. It is reported that CRP up-regulates NF-κB activity in human aortic endothelial cells. These data demonstrate that CRP directly activates the NF-κB signal transduction pathway in HUVECs. Degradation of IκB-α, but not IκB-β, seems to be the major pathway leading to NF-κB nuclear localization and activation induced by CRP. These data support the growing body of evidence indicating that CRP is not only a marker but also an active mediator of atherosclerotic lesion formation.

CD40-CR40L system is proven to be an important mediator of several auto-immune and chronic inflammation diseases. Interruption of CD40-C40L signaling not only reduces the initiation and progression of atherosclerotic lesions in hypercholesterolemic mice in vivo, but also modulates plaque architecture. Moreover, the investigations identify CD40-C40L as a key regulator of this process and recognize it as potentially important atherosclerotic therapeutic target. A role of NF-κB in CD40 expression has recently been proposed for Burkitt lymphoma cells stimulated with Epstein-Barr virus latent membrane protein-1. Our previous study showed that incubation of HUVECs with CRP resulted in a time-and dose-dependent increase in the cell-surface expression of CD40 and CD40L. In the present paper, we found that CRP-induced CD40 gene expression, which was inhibited by PDTC (NF-κB inhibitor) in HUVECs. This result suggests that the induction of CD40 genes by CRP is at least partly mediated by NF-κB activation. Thus, one of the signaling pathways by which CRP activates CD40 expression in HUVECs appears to be via NF-κB activation.

From above we reported a new finding to support the activation of NF-κB induced by CRP in HUVECs. Therefore, the therapeutic modulation of its activation could be of interest in pathological situations. We studied whether lipid-lowering agent, lovastatin, possessed anti-inflammatory properties independent of their lipid-lowering action via CRP-induced the activation of NF-κB and degradation of IκB in HUVECs. Some reports have shown that lovastatin and atorvastatin reduce pro-inflammatory cytokine and chemokine expression in the smooth muscle cells and mononuclear cells through inhibition of NF-κB activity. Furthermore, Atorvastatin, through the inhibition of NF-κB activity and chemokine gene expression, could reduce the inflammation within the atherosclerotic lesion and play a role in the stabilization of the lesion. Our previous studies found that lovastatin and fenofibrate significantly diminished the expression of CD40 and CD40L as well as gelatinase activities (MMP-2, MMP-9) induced by CRP in HUVECs. In the present study, lovastatin significantly diminished NF-κB activation induced by CRP by causing a stabilization of the IκB-α in cellular cytoplasm. The mechanism of inhibition of NF-κB activation by lovastatin seems to be related to the stabilization of subunit IκB-α to prevent IκB degradation. The specific mechanism needs to be found out.

Although further studies are needed, the present data suggest a mechanism that CRP activates the expression of CD40-C40L through NF-κB; this effect can be modulated by pharmacological interventions, particularly lovastatin. Furthermore, CD40 gene expression induced by CRP is at least partly NF-κB-dependent. These results may add a further explanation of the beneficial effects of statins on coronary artery disease in clinical trials.

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REFERENCES

6) Venugopal S. K., Devaraj S., Jialal I., Circulation, 108, 1676—1678
(2003).