C-Reactive Protein Upregulates Receptor for Advanced Glycation End Products Expression in Human Endothelial Cells

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Abstract—The receptor for advanced glycation end products (RAGE) may play an important role in inflammatory processes and endothelial activation, likely to accelerate the processes of coronary atherosclerotic development, especially in diabetic patients. The factors that regulate arterial expression of RAGE are not completely clear. C-reactive protein (CRP) is identified as a key proinflammatory cytokine in patients with atherosclerosis. Therefore, we tested the hypothesis that RAGE expression in endothelial cells can be upregulated by CRP. Human saphenous vein endothelial cells were incubated with human recombinant CRP, free of sodium azide and endotoxin. RAGE protein expression was measured by flow-cytometric analysis and Western blotting. CRP caused a significant increase in RAGE protein expression at a dose as low as 5 \( \mu \)g/mL, with expression peaking at 24 to 48 hours after CRP incubation. The effects of modified monomeric CRP on RAGE protein expression were comparable with that of native pentameric CRP. At the mRNA level, CRP not only increased RAGE gene expression but also attenuated the degradation of RAGE mRNA. Furthermore, RNA interference of RAGE gene expression significantly decreased the level of macrophage chemoattractant protein 1, a key downstream mediator of CRP activity. Therefore, CRP at concentrations known to predict future vascular events upregulates RAGE expression in human endothelial cells at both the protein and mRNA level. Silencing of the RAGE gene prevents CRP–induced macrophage chemoattractant protein 1 activation. These data reinforce the mechanistic links among inflammation, endothelial dysfunction, and atherothrombosis. (Hypertension. 2006;48:504-511.)

Key Words: atherosclerosis ■ endothelial growth factors

Diabetes-associated cardiovascular complications are one of the major causes of patient mortality.1 Numerous epidemiological studies suggest that diabetes can accelerate atherosclerosis and increase the incidence of heart attacks and strokes.2 However, the underlying mechanisms behind this relationship have not been fully elucidated. Recent studies suggest that the receptor of advanced glycation end products (RAGE), a multiligand receptor on vascular cells, may play an important role, especially in diabetic patients, in promoting inflammatory processes and endothelial activation, which accelerate coronary atherosclerotic development. RAGE, a member of the immunoglobulin superfamily of cell surface molecules, was initially isolated from bovine lung; however, subsequent studies from several laboratories demonstrated that RAGE is also expressed on the surface of vascular endothelial and smooth muscle cells.3 As a multiligand cell surface receptor, the endogenous ligands of RAGE are variable and include advanced glycation end products (AGEs), S100/calgranulins, and high mobility group box 1 protein.4 Diabetes-associated hyperglycemia and oxidative stress are the main sources of AGEs, which subsequently activate the RAGE pathway and initiate the inflammatory response.5 Furthermore, RAGE is highly expressed in human atherosclerotic lesions and colocalizes with proinflammatory and pro-oxidative mediators.6

C-reactive protein (CRP), a key proinflammatory cytokine that is highly elevated in atherosclerotic patients, serves not only as a biomarker for the risk of cardiovascular disease but also functions as an active mediator of atherosclerosis by promoting arterial endothelial activation and macrophage recruitment.7 Previous studies from our laboratory revealed that CRP potently downregulates endothelial NO synthase protein expression and destabilizes endothelial NO synthase mRNA, resulting in the decreased release of basal and stimulated NO.8 Furthermore, CRP also induces the expression of macrophage chemoattractant protein 1 (MCP-1), interleukin (IL) 6, IL-8, intercellular adhesion molecule 1, and vascular cell adhesion molecule 1.9–11 Therefore, we hypothesized that CRP may upregulate RAGE expression in endothelial cells and, via this pathway, can magnify vascular inflammation, accelerating the process of atherosclerosis.
RT-PCR

Total cellular RNA was isolated using the RNeasy Mini kit (Qiagen). RT-PCR was performed using the Qiagen One-Step RT-PCR kit. Total RNA served as template for each reaction. For amplification, a primer pair specific for human RAGE was used. The primers used were the following: forward: 5'-GGGCTCTTACACTGC-3' and reverse: 5'-CTCCAGTACTCTCGCCTG-3'. Reverse transcription was performed at 50°C for 30 minutes. For PCR, 35 cycles were used at 94°C for 30 seconds, 52°C for 30 seconds, and 72°C for 30 seconds. The RT-PCR products were visualized on 1% agarose gels using ethidium bromide. GAPDH was amplified as a reference for quantification of RAGE mRNA. The signal intensity of each RAGE band was normalized by that of GAPDH.

mRNA Stability Analysis

The effects of CRP on mRNA stability were also evaluated. For this purpose, after pretreatment with 2.5 μg/mL actinomycin-D (Sigma) for 30 minutes, confluent HSVECs were incubated with CRP (50 μg/mL). Culture medium was removed at 0, 6, 12, and 24 hours after actinomycin-D treatment, and RNA was extracted for RT-PCR.

Small Interfering RNA Assay

Small interfering RNA (siRNA) for RAGE and transfection reagent were purchased from Santa Cruz Inc. siRNA transfection was carried out according to the manufacturer’s protocol for the RAGE-siRNA kit. In brief, 4×10^5 HSVECs were transfected into 6-well plates and cultured in endothelial cell basal medium (Clonetics) supplemented with 10% FBS (Gibco), without antibiotics. After overnight growth, cells at 80% confluence were treated with 0.8 mL of transfection medium (Santa Cruz) with each well containing 10-μmol/L siRNA duplexes and 4.8 μL of transfection reagent. After 7 hours of incubation, 0.8 mL of endothelial cell basal medium containing 20% FBS and antibiotics were added to each well containing transfected cells. Transfection medium was removed 24 hours later, and the cells were washed twice with PBS and then maintained in MCDB-131 complete medium (VEC technologies) for 24 to 72 hours before performing the MCP-1 ELISA. Cell lysates were collected for Western blot analysis to detect the transfection efficiency.

MCP-1 ELISA

For MCP-1 detection, control and RAGE siRNA-transfected HSVECs were incubated for 24 hours in the presence or absence of CRP (50 μg/mL) or LPS (1000 pg/mL). For the positive control, HSVECs were incubated with RAGE antibody 1 hour before being exposed to CRP. Culture supernatant was collected, and the concentration of MCP-1 was assessed using an ELISA kit (R&D Systems) according to the manufacturer’s protocol.

Statistical Analyses

All of the values are presented as mean±SEM. Comparisons between multiple treatment groups were done with the use of 1-way ANOVA followed by a Tukey’s test. Comparisons within 2 treatment groups were analyzed by the t test. Differences were considered significant at P<0.05.
Results

CRP Increases RAGE Protein Expression in a Dose- and Time-Dependent Manner

To examine whether CRP affects RAGE protein expression, HSVECs were treated with CRP at different doses and time points, and RAGE protein was quantified using both flow cytometry and Western blotting. For the dose–response study, HSVECs were treated with CRP at a dose from 5 to 100 μg/mL for 24 hours. Results from the flow-cytometric analysis showed that CRP significantly increased RAGE protein expression at a dose as low as 5 μg/mL. CRP-induced RAGE protein expression was dose dependent, with the maximal effect achieved at 50 μg/mL (Figure 1A and 1B). CRP incubation caused an increase in RAGE protein expression from 2-fold at 5 μg/mL to 5-fold at 50 μg/mL when compared with the untreated control. Consistent with the flow-cytometry results, Western blot analysis also revealed a stepwise increase in RAGE protein expression with increasing CRP concentration, although the magnitude of this increase was lower (Figure 1C and 1D).

To examine the kinetics of CRP-induced RAGE expression, HSVECs were incubated with CRP (50 μg/mL) for ≤72 hours. RAGE protein expression was determined using both flow cytometry and Western blot. Flow-cytometric analysis showed that the increase in RAGE protein expression was first detected after 6 hours and peaked at 24 to 48 hours after CRP treatment. When compared with the control, CRP treatment caused about a 5-fold increase in RAGE expression at 48 hours after incubation (Figure 2A and 2B). The results from Western blot analysis revealed the same trend as flow cytometry, with the initial increase being detected at 6 hours and maximal level of expression being reached at 24 to 48 hours after incubation (Figure 2C and 2D).

CRP Enhances the Binding of BSA AGEs to RAGE

To study whether CRP increases the interaction between RAGE and its endogenous ligand AGEs, binding assays were performed. Flow-cytometric analysis showed that, after incubation with CRP for 24 hours, the relative binding rates of BSA AGEs to RAGE were significantly increased compared with the control groups (Figure 3).

CRP-Induced RAGE Expression Requires Increased mRNA Level and Stability

To investigate whether CRP increases RAGE protein expression by upregulating RAGE mRNA expression, the effect of CRP treatment on RAGE mRNA was determined using RT-PCR. HSVECs were incubated with CRP, at different concentrations, for 12 hours. RAGE mRNA levels were significantly induced by 35%, 51%, 86%, and 45% in response to CRP concentrations of 5, 25, 50, and 100 μg/mL, respectively, when compared with controls (Figure 4A and 4B). Furthermore, upregulation of RAGE mRNA in HSVECs was observed in a time-dependent fashion. In response to 50 μg/mL of CRP, RAGE mRNA was significantly increased by 34%, 114%, 94%, and 63% at 6, 12, 24, and 48 hours, respectively, compared with basal levels (Figure 4C and 4D).

The upregulation of RAGE mRNA in HSVECs, after CRP incubation, was further examined in a time- and dose-dependent fashion using real-time PCR. In response to CRP (50 μg/mL) treatment, RAGE mRNA was significantly increased at each time point, peaking at 24 hours (Figure 5A). Furthermore, the level of RAGE mRNA was significantly increased in a dose-dependent fashion (Figure 5B).

To investigate whether a posttranscriptional regulation mechanism is involved in RAGE expression, we used actinomycin-D at a concentration (5 μg/mL) that inhibits RNA polymerase II activity (mRNA synthesis) but not RNA polymerase I activity (ribosomal RNA synthesis) and, thus, effectively prevents new mRNA synthesis. The stability of RAGE mRNA with or without CRP treatment was measured. Cells were pretreated with actinomycin-D for 30 minutes before exposure to CRP (50 μg/mL) and were harvested for analysis at 6, 12, 24, and 48 hours after CRP treatment. No differences in the mRNA levels

Figure 1. Dose–response effect of CRP on RAGE protein expression. HSVECs were cultured for 24 hours with CRP at a concentration of 5 to 100 μg/mL. Protein levels were assessed by both flow cytometry (A and B) and Western blot (C and D). The red line represents unstained cells, green represents control, and blue represents the CRP group (A). RAGE levels were normalized to the level of β-actin (C). Data represent the mean±SEM (n=5 to 8). *P<0.05 and **P<0.01 vs control.
of RAGE between the control (actinomycin-D alone) and CRP groups at 6, 12, and 24 hours were observed. However, compared with the CRP group, RAGE mRNA in the control group was significantly decreased at 48 hours after actinomycin-D treatment (Figure 6A and 6B). These results suggest that CRP treatment attenuates RAGE mRNA degradation and that post-transcriptional regulation is involved in the CRP-mediated up-regulation of RAGE expression.

Effect of mCRP on RAGE Expression Is Comparable to That of Native CRP

To assess whether native pentameric CRP needs to undergo a structural modification to its monomeric form on the cell membrane to induce RAGE protein and mRNA expression, pentameric CRP was converted to mCRP as described previously. HSVECs were incubated with mCRP (50 μg/mL) for ≤24 hours. RAGE protein levels were then measured using flow cytometry. The observed increase in RAGE protein level by mCRP treatment was comparable to that of native CRP, suggesting that both pentameric CRP and mCRP elevate RAGE expression via the same pathway (Figure 7).

RNA Interference of RAGE Blocks CRP-Induced MCP-1 Upregulation But Not MCP-1 Upregulation Induced by LPS

It has been demonstrated that both CRP and LPS upregulate MCP-1 expression, a key chemokine involved in the inflammatory response of atherosclerosis. To determine whether CRP induces MCP-1 levels via the RAGE pathway, HSVECs were transfected with RAGE-specific siRNA or transfection reagent alone in the absence and presence of CRP (50 μg/mL) or LPS (1 ng/mL) for 24 hours. RAGE siRNA reduced RAGE protein expression by ≈70% in both the control and the CRP groups (Figure 8). To determine whether this resulted in inhibition of MCP-1 synthesis, ELISA was performed. Interference RAGE mRNA prevented CRP-induced MCP-1 expression but not LPS-induced expression (Figure 9). Moreover, there is a correlation between the downregulation of MCP-1 protein and the decrease of RAGE protein level by siRNA in both the control and CRP groups, implying that RAGE may play an essential role in the CRP-induced MCP-1 response.

Discussion

Both RAGE and CRP levels are elevated in atherosclerosis, suggesting that these 2 proteins are involved in its progression, especially in diabetic patients. However, it is not entirely clear as to whether there is an interaction between these 2 proteins. In the present study, we first demonstrated that CRP upregulates RAGE expression in HSVECs in a dose- and time-dependent manner. Second, CRP enhances the binding
ability of RAGE with its endogenous ligands. Third, the RAGE mRNA level is elevated by CRP treatment in a dose- and time-dependent manner, suggesting that CRP increases RAGE protein through the upregulation of RAGE mRNA expression. Furthermore, CRP delayed RAGE mRNA degradation, implying that CRP may serve as a posttranscriptional regulator of RAGE expression. Fourth, compared with native, pentameric CRP, mCRP, which undergoes transformation, has the same effect on RAGE expression. Fifth, by blocking RAGE protein expression by using interfering RAGE mRNA, MCP-1 levels are restored. Because MCP-1 is a downstream mediator of CRP, this observation suggests that CRP induces MCP-1 expression in part via the RAGE signaling axis. However, the further signaling steps and proteins involved in this molecular pathway were not examined with the current set of experiments.

Diabetes and its observed ability to accelerate the progression of atherosclerosis cannot simply be explained by the traditional cardiac risk factors, such as smoking, hypertension, and hyperlipidemia. Recently, the receptor of advanced glycation end products was identified as one of the underlying mechanisms that may assist in explaining this observation. As a multiligand receptor on vascular cells, RAGE has been shown to exert proinflammatory effects, including endothelial activation, which promote atherogenesis. Previous studies have shown that in apolipoprotein E–deficient mice, enhanced expression of RAGE was observed in diabetes-associated atherosclerotic lesions and that blockage of RAGE seemed to suppress the further progression of these lesions.

CRP, first described as a biomarker for inflammation, seems to serve as a prominent partner in promoting endothelial dysfunction and subsequent atherosclerosis. It has been demonstrated that, in vitro, CRP at concentrations ≥5 μg/mL, has significant proinflammatory effects on endothelial cells, an observation that is consistent with large, prospective studies that show an increased risk of cardiac events in patient with CRP levels >5 μg/mL. Previous studies in

Figure 4. Dose- and time-dependent effects of CRP on RAGE expression. HSVECs were treated for 12 hours with 5 to 100 μg/mL of CRP (A and B) or incubated ≤48 hours with 50 μg/mL of CRP (C and D). Total cellular RNA was isolated and analysis by RT-PCR performed. RAGE mRNA levels were normalized to the levels of GAPDH mRNA (A and C). Data represent the mean±SEM (n=4). *P<0.05 and **P<0.01 vs control.

Figure 5. Dose- and time-dependent effects of CRP on RAGE mRNA induction. HSVECs were treated for 24 hours with 5 to 100 μg/mL of CRP (A) or incubated ≤48 hours with 50 μg/mL of CRP (B). Total cellular RNA was isolated and analysis done by both reverse transcriptase and real-time PCR. RAGE mRNA levels were extrapolated from a standard curve constructed by serial dilution of an internal control and normalized to the level of GAPDH mRNA. Data represent the mean±SEM (n=4). *P<0.05 vs control.
our laboratory also show that CRP stimulates endothelin-1 and IL-6 release from endothelial cells; increases the expression of intercellular adhesion molecule 1, vascular cell adhesion molecule 1, and E-selectin; and upregulates nuclear factor κB, which facilitates the transcription of numerous proatherosclerotic genes. Although some in vivo studies conducted in human CRP transgenic mice have suggested that human CRP does not play a proatherogenic or prothrombotic role,23,24 others have demonstrated that this CRP transgenic mouse model exhibits increased rates of atherosclerosis.25 It is difficult to determine which of the reports is valid or invalid, because the model itself encounters several problems because human CRP is a foreign antigen in the mouse, with many uncertainties concerning its functional role in the immune system of these animals.7 Furthermore, an in vivo role for CRP in the development of endothelial dysfunction and inflammation in humans was suggested by a set of elegant experiments in which a marked activation of inflammation and coagulation after the infusion of CRP in male volunteers was observed.26

It was demonstrated previously that native pentameric CRP needed to undergo a structural change on cell membrane binding in order for endothelial activation to occur.12,27 It has also been demonstrated that mCRP, which loses the pentameric symmetry of native CRP, could be detected in normal blood vessels.28 Khreiss et al26 showed that modified mCRP, used at lower concentrations and at shorter periods of time, could cause similar inflammatory responses as native pentameric CRP. However, experiments conducted by Devaraj et al28 suggest that native pentameric CRP displays more potent proatherogenic activities, such as the elevation of plasminogen activator inhibitor 1 and IL-8 levels in human aortic endothelial cells, than that of mCRP. In this report, we present data suggesting that there is no significant difference between native and modified CRP in terms of inducing RAGE expression, suggesting that both forms of CRP share the same intracellular pathway to stimulate RAGE expression, although they may bind at different sites.

There have also been some criticisms recently surrounding the use of commercial CRP preparations for in vitro studies. Recent publications have reported that the effect of commercial CRP, which contains some biologically active contaminants, may be explained by the presence of NaN3 and endotoxin.29–31 However, subsequent studies demonstrate that azide and LPS-free CRP elicit similar effects on endothelial activation. Indeed, Singh et al32 have demonstrated recently that endotoxin-purified, azide-free CRP inhibits NO release and decreases tissue plasminogen activator in aortic endothelial cells. The role of LPS and NaN3 toward the effects of CRP on endothelial activation have been laid to rest by observations in endothelial cells indicating that: (1) monoclonal antibodies to CD32 inhibit the proinflammatory

Figure 6. Effect of actinomycin-D on RAGE mRNA levels in HSVECs. HSVECs were pretreated with actinomycin-D (5 μg/mL) for 30 minutes and subsequently incubated with or without CRP (50 μg/mL) for 6, 12, 24, or 48 hours (A). The graph shows quantification of remaining RAGE mRNA by densitometric scanning (B). Data represent the mean±SEM (n=4). *P<0.05 vs control.

Figure 7. Comparison of the effects of mCRP and native pentameric CRP on RAGE protein expression. HSVECs were treated with CRP or mCRP (50 μg/mL) for 24 hours. RAGE protein expression was detected by flow cytometry. Data represent mean±SEM (n=6 to 8). *P<0.05 vs control.
effects of CRP; (2) LPS (50 and 1000 pg/mL) had no effect on CRP-induced tissue plasminogen activator activity; (3) polymyxin B did not affect the effects of CRP, whereas boiling and/or trypsinization abrogated the effect; and (4) the proinflammatory effects of CRP were observed in cells that had been preabsorbed to anti-CRP IgG-coated plates but not to plates without anti-CRP IgG. These observations argue strongly in favor of a specific proatherosclerotic effect of CRP, not attributable to contaminants or NaN₃. Likewise, in the present study, all of the reagents and media used for cell culture were free of endotoxin (≤0.05 ng/mL), and NaN₃-free CRP was specially ordered from Trichem Resources Inc and used in all of the experiments. Moreover, modified CRP was dialyzed 3 times during its preparation. The consistent results obtained from either azide-free CRP or dialyzed mCRP support the conclusion that CRP is the sole cause for the observed increase in RAGE expression.

In the present study, a prominent increase in RAGE protein expression was observed by flow cytometry when compared with the amount measured by Western blotting. This observed difference could possibly be explained by the different sensitivities of each assay. For one, the signals detected by Western blotting are based on the ratio of RAGE protein to total protein extracted, whereas the RAGE signals observed with flow cytometry were detected on the surface of each cell membrane and subsequently amplified by a fluorescent-conjugated secondary antibody. Overall, we feel that flow cytometry is the more sensitive test, and, therefore, the magnitude of increased RAGE expression detected by flow cytometry is greater. Furthermore, differences in mRNA RAGE levels were observed when the results of RT-PCR were compared with real-time PCR. Compared with RT-PCR, the RAGE mRNA level peaked at 24 hours after CRP incubation with a much greater amplitude in the real-time PCR groups. This is likely secondary to the fact that RT-PCR is a semiquantification method whereas real-time PCR is able to detect the total amount of cDNA by using double-stranded DNA-specific SYBR green dye.

Previous reports have demonstrated that CRP increases MCP-1 levels both in vitro and in vivo. We propose that RAGE functions as an amplifying factor in the propagation of the vascular inflammatory response, rather than serving as an initiating factor, in the progression of atherosclerotic disease in diabetic patients. In the current study, using RAGE-specific siRNA to block RAGE expression, we were able to restore CRP-induced MCP-1 expression. Although LPS (1 ng/mL) can increase MCP-1 levels, the amplitude is much lower than that induced by CRP. Furthermore, blocking RAGE expression does not affect LPS-induced MCP-1 expression. These results suggest that CRP elevates the level of MCP-1 by increasing RAGE expression, providing it with an avenue to amplify its proatherogenic effects.

**Perspectives**

The current study, for the first time, demonstrates that CRP, at concentrations known to predict future cardiovascular events, upregulates RAGE expression at both the protein and mRNA level in human endothelial cells. CRP also enhances
RAGE ligand binding. mCRP, which undergoes a transformation from its native pentameric form, exerts the same effect on RAGE expression as native CRP, suggesting that both forms of CRP share the same intracellular pathway, which leads to the stimulation of RAGE expression. Silencing of RAGE expression restores CRP-induced MCP-1 activation, suggesting that CRP increases MCP-1 levels via the RAGE pathway. Although not examined in the current study, elucidation of the molecular signaling steps involved in this pathway or inhibition of RAGE itself may serve as novel therapeutic targets for limiting CRP-induced atherogenesis, especially among patients with diabetes or impaired glucose tolerance. Overall, the present study provides a further mechanistic link among the triad of vascular inflammation, endothelial dysfunction, and atherothrombosis.34

Sources of Funding

This work was supported in part by the GuangDong Natural Science Foundation of People’s Republic of China (04009589) and a grant in aid from CIHR (S.V.).

Disclosures

None.

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*Hypertension*. 2006;48:504-511; originally published online July 17, 2006;
doi: 10.1161/01.HYP.0000234904.43861.f7

*Hypertension* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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