C-Reactive Protein Adversely Alters the Protein–Protein Interaction of the Endothelial Isoform of Nitric Oxide Synthase

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BACKGROUND: C-reactive protein (CRP) inhibits the activity of the endothelial isoform of nitric oxide synthase (eNOS) via uncoupling of the enzyme both in vitro and in vivo. eNOS activity appears to be related in part to its interaction with other cellular proteins, including heat shock protein 90 (Hsp90), caveolin-1, and porin. In this study, we examined the effect of CRP treatment of human aortic endothelial cells (HAECs) on eNOS interaction with caveolin-1, Hsp90, and porin.

METHODS: We incubated HAECs with CRP (0, 12.5, and 25 mg/L) for 1, 6, or 24 h and assessed the interaction of these proteins with eNOS by immunoprecipitation and western blotting.

RESULTS: CRP treatment (12.5 and 25 mg/L) of HAECs for 24 h significantly increased eNOS binding to caveolin-1 (40% and 54% increase, respectively; *P* < 0.05) and decreased binding to Hsp90 (33% and 66% decrease, respectively; *P* < 0.05). CRP (25 mg/L) also significantly decreased the binding of porin to eNOS (11% decrease, *P* < 0.05). Similar results were seen when HAECs were treated with CRP for 6 h.

CONCLUSIONS: These negative protein–protein interactions of eNOS were able to partly explain the CRP-induced decreases in the activity of this critical enzyme, which caused endothelial dysfunction.

C-reactive protein (CRP),2 a member of the pentraxin family, is the prototypic marker of inflammation in humans (1) and is a valid marker of cardiovascular risk (2). Mounting data also support a role for CRP in atherothrombosis (3, 4). Several studies have shown a significant relationship between CRP concentration and endothelial dysfunction (3, 4). The endothelial isoform of nitric oxide synthase (eNOS) is critical for maintaining endothelial function via its product, NO, which inhibits platelet aggregation, inhibits monocyte adhesion, and promotes vasomotion by relaxing smooth muscle cells (5). The production and activity of eNOS are finely regulated in a complex fashion at various levels, including gene expression, substrate availability, cofactors, phosphorylation, posttranslational modifications (including myristoylation), cellular localization, and protein–protein interactions (5–7). CRP has previously been shown to inhibit eNOS activity and bioactivity (8, 9). In addition, the mechanism of inhibition appears to occur via uncoupling of the enzyme through increases in reactive oxygen species that cause decreased eNOS dimerization and phosphorylation of the eNOS Ser 1177 residue (10). Furthermore, numerous groups have shown that CRP impairs endothelial vasoreactivity in vivo (11). To date, eNOS has been shown to interact directly with several proteins, including heat shock protein 90 (Hsp90), calmodulin, dynamin-2, caveolin-1, the intracellular domains of certain G protein–coupled receptors, and porin, a voltage-dependent anion/cation channel (5–7, 12–15). The most important interactions for activity appear to relate to its association with caveolin-1 (decreased activity) and Hsp90 (increased activity) (5–7). Despite reports from various groups of in vitro and in vivo inhibition of eNOS, there is a paucity of data on the protein–protein interactions of eNOS after CRP treatment (11). Hence, our focus in this study was on the effect of CRP on eNOS interaction with caveolin-1, Hsp90, and porin.

CRP was purified from human ascitic/pleural fluids as previously described (16, 17). For all experiments, we used both boiled CRP and CRP preadsorbed on CRP antibody–coated plates as controls. Furthermore, the addition of polymixin B (5 mg/L) (16) did not alter any of the effects observed with CRP.

Human aortic endothelial cells (HAECs) were cultured in endothelial cell growth medium with supplements (EGM-MV) as described previously (8, 10). When confluent, the cells were exposed for 1, 6, and 24 h to either (a) serum-free media (control) or (b) serum-free media containing different CRP levels (3, 6, 12.5, or 25 mg/L). cGMP levels were assayed as previously described (9). No significant differences in cell cytotoxicity were observed with CRP treatment.

Control and CRP-treated HAECs were lysed with IP Lysis/Wash Buffer (Pierce Biotechnology) and

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2 Nonstandard abbreviations: CRP, C-reactive protein; eNOS, endothelial isoform of nitric oxide synthase; Hsp90, heat shock protein 90; HAEC, human aortic endothelial cell.
Concluded, total protein in the cell lysates was assessed by bicinchoninic acid assay (10). Immunoprecipitation studies were then performed. Equal amounts of protein were pre cleared and then incubated overnight at 4 °C with 2 μL eNOS mouse monoclonal antibody (Santa Cruz Biotechnology) per 100 μg total protein. This mixture was then incubated with Protein A/G agarose beads (Pierce Biotechnology) overnight at 4 °C, with gentle shaking. After 5 washes with IP Lysis/Wash Buffer, immune complexes were eluted by boiling in sample buffer from IP kit (Pierce Biotechnology). Samples were pre cleared by centrifugation, loaded onto a precast 8%–16% Precise™ Protein Gel (Pierce Biotechnology), subjected to electrophoresis, and then transferred to Invitrolon™ polyvinylidene fluoride membranes (Invitrogen) according to manufacturer recommendations. Blots were hybridized overnight at 4 °C with the appropriate antibody: eNOS mouse monoclonal antibody (1:200 dilution; Santa Cruz Biotechnology); caveolin-1 rabbit polyclonal antibody (1:200 dilution; Santa Cruz Biotechnology); Hsp90 rabbit polyclonal antibody (1:200 dilution; Santa Cruz Biotechnology); porin mouse monoclonal antibody (3 mg/L; Abcam), or CRP rabbit polyclonal antibody (1:200 dilution; Sigma-Aldrich). After washing and incubation for 1 h at room temperature with the respective horseradish peroxidase-conjugated goat antimouse or goat antirabbit antiserum secondary antibodies (1:4000 dilution; Santa Cruz Biotechnology), proteins were detected by chemiluminescence by using manufacturer’s directions (Pierce enhanced chemiluminescence kit; Pierce Biotechnology). The densitometric analysis of band intensities was performed with the NIH’s Image J64 Software, available online (rsbweb.nih.gov/ij/download.html). All experiments were done in duplicate on 3–6 occasions. Data are presented as the mean (SD). The analysis of mean densitometric ratios of caveolin, Hsp90, and porin to eNOS protein respectively was performed by using NIH’s Image J64 software (see URL above). A P value <0.05 was considered statistically significant.

As shown in a representative blot in Fig. 1A, incubation of HAECs with CRP at concentrations ≥12.5 mg/L for 24 h produced increased eNOS binding to caveolin-1 and decreased binding to Hsp90. Densitometric analyses of at least 3 consecutive experiments in duplicate revealed that compared with HAECs not treated with CRP, binding of eNOS to caveolin-1 was increased 40% and 54% at CRP concentrations of 12.5 mg/L and 25 mg/L, respectively, and eNOS binding to Hsp90 was decreased 33% and 66%, respectively. These protein interactions were statistically significant (Fig. 1B). Furthermore, these effects were not seen with boiled CRP or after CRP pretreatment with an anti-CRP antibody, and they were not abrogated with polymixin B (data not shown).

We also examined these protein–protein interactions after only 1 and 6 h of CRP treatment. No significant effects were seen within 1 h of treatment.
but similar effects were seen after 6 h of CRP treatment. In addition, we noted a significant decrease (11%) in binding of porin to eNOS in the presence of 25 mg/L CRP, compared with the control (P < 0.05; see Fig. 1 in the Data Supplement that accompanies the online version of this Brief Communication at http://www.clinchem.org/content/vol56/issue8). Interestingly, CRP was associated with eNOS (Fig. 1C).

As reported previously (9), we noted a significant decrease in eNOS bioactivity at both 6 h and 24 h when we assayed cGMP concentrations (cGMP release at 24 h: control, 796 (91) fmol/mg cell protein; 12.5 mg/L CRP, 446 (177) fmol/mg cell protein; 25 mg/L CRP, 347 (99) fmol/mg cell protein) (n = 3; P < 0.0001).

CRP is an accepted marker of cardiovascular risk and appears to play a role in atherothrombosis. CRP inhibits eNOS activity, both in vitro and in vivo (11). At a posttranslational level, eNOS function has been shown to be dependent on protein–protein interactions. NO production is determined by an eNOS signaling complex that consists of the enzyme and at least 6 different proteins, including Hsp90, calmodulin, dynamin-2, caveolin-1, the intracellular domains of certain G protein–coupled receptors, and porin, a voltage-dependent anion/cation channel. The docking of these proteins is able to increase or decrease the enzyme’s activity. It appears that the 2 most critical interactions are with caveolin-1 (decreases activity) and Hsp90 (increases activity). We focused on the interactions between eNOS and caveolin-1, Hsp90, and porin. Caveolin-1 is a 21-kDa integral membrane protein and one of the major structural components of caveolae in most mammalian cells, along with caveolin-2 and caveolin-3. Caveolae, small plasma membrane invaginations involved in the regulation of various signaling cascades, play a crucial role in regulating NO availability (18). The binding of eNOS to caveolin-1 inhibits eNOS activity. In fact, eNOS is active only in its dimeric configuration, and the eNOS–caveolin-1 heteromeric complex adversely affects eNOS dimerization and activity, irrespective of the presence or absence of agonist stimulation (12–15).

Hsp90 is a 90-kDa cytosolic protein that belongs to a highly conserved family of stress proteins that are produced in all eukaryotic cells. It is a molecular chaperone that mediates the folding and maturation of proteins (19). Hsp90 has been shown to bind eNOS, enhancing its activity. The molecular mechanism of this action seems to be based on the ability of Hsp90 to allosterically activate/modulate eNOS (12). The conformational change induced by eNOS binding to Hsp90 stabilizes the dimeric form of eNOS, leading to increased enzyme activity, as demonstrated by enhanced agonist-induced NO production (7). Porin is an integral β-barrel membrane protein of about 35 kDa that forms a wide pore in the membrane, which allows the transport of anions, cations, and larger metabolites (15). Porin and eNOS have recently been shown to interact directly and specifically. Porin binding to eNOS enhances eNOS activity, but the molecular mechanism of this enhancement has not been elucidated completely (15). As a voltage-dependent membrane channel, porin can regulate the Ca2+ concentration, which regulates eNOS activity as well as substrate and cofactor delivery in the proximity of eNOS (15). In summary, the interaction of eNOS with Hsp90 and porin enhances eNOS activity, whereas its association with caveolin-1 inhibits its activity.

In the present study, we found that the impairment of endothelial function exerted by CRP can also be related to its ability to alter eNOS protein–protein interaction toward a less active state of the enzyme. This function is in addition to increasing the reactive oxygen species that cause uncoupling of eNOS and decreased phosphorylation of eNOS (10). CRP promotes both increased eNOS binding to caveolin-1 and decreased binding to Hsp90 and porin. Although these effects were apparent at both 6 h and 24 h, they appeared more pronounced at 24 h. The association of CRP with the eNOS complex is very interesting and suggests that by binding directly to the enzyme, CRP determines the interaction of eNOS with other proteins. In conclusion, the present report makes the novel observation that CRP alters the eNOS protein–protein interaction in a negative manner. This is an additional mechanism for CRP inhibition of eNOS leading to endothelial dysfunction.

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