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Original article

C-reactive protein decreases endothelial nitric oxide synthase activity via uncoupling

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Abstract

C-reactive protein (CRP), a cardiovascular risk marker, induces endothelial dysfunction. We have previously shown that CRP decreases endothelial nitric oxide synthase (eNOS) expression and bioactivity in human aortic endothelial cells (HAECs). In this study, we examined the mechanisms by which CRP decreases eNOS activity in HAECs. To this end, we explored different strategies such as availability of tetrahydrobiopterin (BH4)-a critical cofactor for eNOS, superoxide (O_2^-) production resulting in uncoupling of eNOS and phosphorylation/dephosphorylation of eNOS. CRP treatment significantly decreased levels of BH4 thereby promoting eNOS uncoupling. Pretreatment with sepiapterin, a BH4 precursor, prevented CRP-mediated effects on BH_4 levels, superoxide production as well as eNOS activity. The gene expression and enzymatic activity of GTPCH1, the first enzyme in the de novo biosynthesis of BH_4, were significantly inhibited by CRP. Importantly, GTPCH1 is known to be regulated by cAMP-mediated pathway. In the present study, CRP-mediated inhibition of GTPCH1 activity was reversed by pretreatment with cAMP analogues. Furthermore, CRP-induced O_2^- production was reversed by pharmacologic inhibition and siRNAs to p47 phox and p22 phox. Additionally, CRP treatment significantly decreased the eNOS dimer: monomer ratio confirming CRP-mediated eNOS uncoupling. The pretreatment of cells with NO synthase inhibitor (*N*-nitro-L-arginine methyl ester [L-NAME]) also prevented CRP-mediated O_2^- production further strengthening CRP-mediated eNOS uncoupling. Additionally, CRP decreased eNOS phosphorylation at Ser1177 as well as increased phosphorylation at Thr495. CRP appears to mediate these effects through the Fcγ receptors, CD32 and CD64. To conclude, CRP uncouples eNOS resulting in increased superoxide production, decreased NO production and altered eNOS phosphorylation.

Keywords: CRP; Endothelial nitric oxide synthase; Oxidative stress; Tetrahydrobiopterin; Uncoupling

1. Introduction

Inflammation plays a critical role in atherogenesis. C-reactive protein (CRP) is a prototypic marker of inflammation, and has been shown in numerous prospective studies, to predict cardiovascular events (CVE) [1,2]. While CRP is a risk marker, much data are evolving to suggest that CRP also promotes atherothrombosis [3]. To date, it has been shown that in monocytes, CRP induces the production of inflammatory cytokines and promotes monocyte chemotaxis, reactive oxygen species and tissue factor expression [4]. In endothelial cells, CRP increases the expression of cell adhesion molecules, monocyte-chemotactic protein-1 and endothelin-1, plasminogen activator inhibitor-1 and inflammatory cytokines, and decreases prostacyclin release as well as tissue plasminogen activator activity [5]. In addition, 2 groups [6,7] have shown that CRP causes down-regulation of endothelial nitric oxide synthase (eNOS) by decreasing eNOS activity and NO bioactivity. Also, three groups have independently shown that the human CRP transgenic (Tg) mice exhibit decreased eNOS activity in vivo [8–10] corroborating the inverse correlation between CRP and endothelium vasoreactivity in human subjects [11–13].

Endothelial NOS is subject to various forms of regulation including availability of cofactors (tetrahydrobiopterin [BH4]) and substrates, subcellular localization, protein–protein interactions and phosphorylation [14,15]. Furthermore, reduced BH4

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availability has been shown to lead to eNOS uncoupling resulting in the generation of superoxide instead of NO [14,15]. The levels of BH4 are principally regulated through 2 distinct pathways [16]: a de novo synthetic pathway, in which GTP is a required precursor, and a salvage pathway. We also investigated the effect of CRP on the mRNA expression and activity of GTPCH1, the first enzyme in the de novo biosynthesis of BH4.

Impaired endothelium-derived NO bioactivity may also be due, in part, to generation of reactive oxygen species [17], which can be produced by a variety of cellular enzymes, including NADPH oxidase, xanthine oxidase and mitochondrial respiratory chain enzymes. Importantly, the NADPH oxidases seem to be upstream of the activation of other ROS producing enzymes [17]. NADPH oxidase consists of several membrane-bound subunits and cytosolic subunits. On activation, some components are phosphorylated and translocated to the membrane and form the catalytically active oxidase. In this context, CRP has been shown to inhibit endothelium-dependent NO-mediated dilation in coronary arterioles by producing superoxide from NAD(P)H oxidase via p38 kinase activation [18]. Our group has previously shown that CRP induces the production of superoxide anion in HAECs [5]. Also, Kobayashi et al. [19] reported that the incubation of cultured smooth muscle cells with CRP resulted in enhanced p22 phox protein expression and in the generation of intracellular ROS. Also, CRP colocalized with the p22 phox subunit of NADPH oxidase in vascular SMCs. Thus, it is plausible that CRP-induced oxidative stress could lead to eNOS uncoupling in cultured ECs, although this has not been demonstrated experimentally.

Thus, it is evident that impaired endothelium-derived NO bioactivity is due to either the generation of reactive oxygen species [17] or decreased BH4 availability [15,16] resulting in eNOS uncoupling. Since the mechanism by which CRP decreases eNOS in HAECs has not been elucidated, we explored these possibilities in CRP-mediated eNOS inhibition including BH4 availability, superoxide production, phosphorylation and dimerization of eNOS in HAECs.

2. Materials and methods

CRP was purified from human ascitic/pleural fluids as described previously [20]. Recently we have shown that our in-house purified, dialyzed CRP inhibits eNOS activity in TLR4 knocked down cells providing further cogent data that CRP-mediated eNOS inhibition is not due to endotoxin contamination [21].

2.1. Cell culture and treatment

HAECs (Cambrex) were grown in endothelial medium containing growth supplements (EGM-MV) and used between passages 3 and 5. For the initial experiments, cells were grown in 12 well plates. Upon reaching 80% confluency, the cells were incubated with CRP (25 and 50 μg/ml) for 12 h. However, for all other experiments involving mechanistic insights, CRP at 25 μg/ml concentration was used.

2.2. eNOS mRNA expression, enzymatic activity and NO bioactivity

eNOS mRNA expression was assessed as reported previously [6] using eNOS specific primers and using GAPDH as an internal control. The enzymatic activity was assessed by measuring the conversion of ^{14}C-L-arginine to ^{14}C-L-citrulline for 60 min in HAEC lysates as reported previously [6]. NO bioactivity was assayed by measuring secreted cyclic GMP levels as reported previously [6]. The precision of NOS activity and cGMP assays was good (coefficient of variation <5%).

2.3. Assay of eNOS dimer/monomer

eNOS dimer (active state) and monomer (inactive state) forms were assayed using low temperature SDS-PAGE run at 70 V on 6% mini-gels using non-boiled cell lysates and reducing sample buffer as described [22].

2.4. CRP, BH_4 levels and eNOS uncoupling

eNOS uncoupling has been linked to reduced tetrahydrobiopterin (BH_4) availability [23,24], thus we tested the hypothesis that CRP-mediated eNOS uncoupling is due to decreased intracellular BH4 levels. The cells were grown in 10 cm dishes and treated with CRP (25 μg/ml) for 12 h. To investigate if BH_4 supplementation through the salvage pathway [16] prevents the CRP-mediated effect, the cells were treated with or without sepiapterin (a BH4 precursor-100 μM-Sigma) 1 h prior to CRP treatment. At the end of incubation, the cells were washed twice with cold PBS. The cells were scraped in PBS-EDTA (2.5 mM) and centrifuged at 10,000 rpm for 5 min at 4 °C. The pellets were immediately frozen in liquid nitrogen. BH4 quantification was performed by HPLC with electrochemical detection (EC-HPLC) as previously described [14]. Intracellular concentrations were calculated using authentic BH4 (10–100 nM) as standards and normalized to protein content. Also, eNOS activity as well as cGMP release was examined as described previously [6].

2.5. GTP cyclohydrolase 1 mRNA expression and activity

BH4 is synthesized from guanosine-triphosphate (GTP) via de novo pathway by GTP cyclohydrolase I (GTPCH1) enzyme which is the first enzyme in this biosynthetic pathway. Thus, we also examined the effect of CRP on GTPCH1 gene expression and its activity in HAECs. The cells were treated with CRP for 12 h in serum free medium. At the end of the treatment, GTPCH1 mRNA expression was assessed by RT-PCR using GTPCH1 specific primers (Integrated DNA Technology) (forward: 5′-GCC ATG CAG TTC TTC ACC AA-3′ and reverse: 5′-AGG CTT CCG TGA TTG CTA CA-3′) and using GAPDH as an internal control as used previously [6]. For GTPCH1 activity measurement, the cells were harvested with PBS-EDTA (2.5 mM) and the pellets were stored in −80 °C until analysis. GTPCH1 activity was assayed [25] based on the quantitation of neopterin [D-*erythro*-neopterin] by ELISA (Alpco) after