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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 (O₂⁻) 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 BH₄ 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 BH₄, was 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 O₂⁻ 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 O₂⁻ 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, altered eNOS phosphorylation.

Keywords

CRP; endothelial nitric oxide synthase; oxidative stress; tetrahydrobiopterin

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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 is 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, 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, 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 downregulation 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 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.

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].

Cell Culture and treatment

HAECs (Cambrex) were grown in endothelial medium containing growth supplements (EGM-MV) and used between passages 3-5. 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.

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%).

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].

CRP, BH₄ levels and eNOS uncoupling

eNOS uncoupling has been linked to reduced tetrahydrobiopterin (BH₄) 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 hrs. To investigate if BH₄ 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 hr 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].

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 hrs 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 conversion of enzymatically formed D-erythro-7,8-dihydroneopterin triphosphate into D-erythroneopterin at 37°C for 60 minutes followed by sequential reaction

of iodine oxidation and dephosphorylation. Intra- and inter-assay CV for neopterin measurement was <10%.

CRP and the involvement of cAMP-mediated pathway in GTPCH1 and eNOS inhibition

Since GTPCH1 activity is known to be regulated by cAMP-mediated pathway [26], we first explored the involvement of this pathway in CRP-mediated GTPCH1 activity inhibition. The cells were treated with two different cAMP analogues (Calbiochem), Br-cAMP and db-cAMP (0.5 mM each separately) 1 hr prior to CRP treatment followed by the measurement of GTPCH1 activity. We also measured the effect of CRP treatment in HAECs pretreated with or without cAMP analogues on eNOS activity and bioactivity as described above.

Cell Transfections

HAECs at 70% confluency were transfected with siRNA (Ambion) for p47 phox, p22 phox or control using siPORTAmine transfection reagent (Ambion) as described previously [5]. Transfection efficiency was ~70% as determined by RT-PCR for p47phox and western blotting for p22 phox. 48 hours after transfection, the cells were treated with CRP (25 µg/ml). The cells and supernatants were collected and stored at -80°C until analysis for citrulline and cGMP measurement respectively.

Measurement of O₂⁻ production

HAECs were treated with CRP (25 µg/ml) in phenol red free endothelial medium for 6 hrs. In the last hour of incubation, O₂⁻ production was measured by the superoxide dismutase (SOD)-inhibitable reduction of acetylated ferricytochrome C (Sigma) as reported previously [27] with or without SOD (100µg/ml). Results were expressed as nmoles/min/mg protein. Since NADPH oxidase is reported to be upstream of the activation of other ROS producing enzymes, we first explored its role in CRP- induced superoxide production using two strategies: pharmacologic inhibition (by using DPI- a common NADPH oxidase inhibitor) as well as RNA interference (by using siRNA to p47 phox subunit and p22 phox). Since NADPH oxidase is known to be regulated upstream by PKC pathway, we also tested the effect of various PKC inhibitors (bisindolylmaleimide, chelerythrin and Calphostin C [10 uM each] on CRP-mediated superoxide release. Furthermore, allopurinol (10µM) as an inhibitor to xanthine oxidase (XO) [18] and various inhibitors to mitochondrial respiratory chain enzymes (100uM AOAC, 5uM rotenone, 10uM TTFA, 5uM HOCA) [27] were used to determine the role of XO and enzymes of the mitochondrial respiratory chain respectively in superoxide generation in HAECs. Furthermore, it has been reported that eNOS itself synthesizes O₂⁻ instead of NO [28]. Thus, we used NO synthase inhibitor (L-NAME- 1mM) as described [28] prior to CRP treatment to examine if eNOS contributes to CRP-induced superoxide production. In all experiments, cells were treated with various inhibitors 1 hr prior to CRP treatment. In addition, we tested the effect of sepiapterin (100 uM) in CRP- mediated O₂⁻ production to further explore if superoxide production was as a result of BH4 depletion in HAECs. Furthermore, we also tested intracellular superoxide using dihydroethidium (DHE) fluorescence. The cells following CRP treatment were incubated with DHE (2uM) for 30 min followed by observation under fluorescence microscope.

CRP-mediated eNOS downregulation via eNOS uncoupling

In separate experiments, we explored the role of CRP-induced oxidative stress leading to eNOS uncoupling. We again tested the effect of NADPH oxidase inhibition using strategies as mentioned above (pharmacologic as well as siRNA interference of p47 phox) on CRP-mediated eNOS downregulation. Also, the inhibition of xanthine oxidase, mitochondrial respiratory chain enzymes, and pretreatment with sepiapterin was tested on CRP-mediated eNOS downregulation. Since the depletion of L-Arg due to enhanced arginase activity can contribute

to endothelial dysfunction, we also tested the effect of arginase inhibitor [29 (BEC- 5 μ M) on CRP-mediated eNOS inhibition.

Phosphorylation studies, immunoprecipitation and western blot

HAECs were stimulated with VEGF (100 ng/ml) for 0-15 minutes after CRP treatment (0 and 25 μ g/ml) for 12 hrs. The cells were lysed in ice cold lysis buffer (Cell Signaling Technology) containing 1% Triton X-100, 25 mM sodium deoxycholate, 150mM NaCl, 20mM Tris, pH 7.4, 1mM EDTA, 200 μ M sodium orthovanadate, 2.5mM sodium pyrophosphate, 1mM NaF, 1mM phenylmethanesulfonyl fluoride, and 2.5% protease inhibitor cocktail. The cell suspensions were sonicated and centrifuged at 10,000 g for 10 min/4°C. Supernatant protein was quantitated. eNOS was immunoprecipitated after preclearing of the cell lysates with a mixture of Protein A/G Sepharose 4B (Sigma Chem Co). Proteins in the immunoprecipitate were heated with SDS-PAGE sample buffer and electrophoresis was carried out as described earlier [5]. Membranes were blocked with 5% milk and then incubated with anti-eNOS, anti-phospho-Ser1177, anti-phospho-Thr 495 in 5% BSA/TBS. After washing and incubating with specific HRP-conjugated secondary antibodies, the membranes were developed with enhanced chemiluminescence (ECL-Amersham-Pharmacia). To reprobe western blots with alternative primary antibodies, nitrocellulose membrane was incubated with stripping buffer (Roche). After extensive washing, the membranes were again incubated in blocking buffer and subsequently with the primary antibody. In separate experiments, we also performed western blotting to examine if CRP treatment results in p22phox upregulation and also to subsequently confirm transfection efficiency.

CRP and the role of Fc γ Receptors in eNOS downregulation

We have previously shown that CRP mediates its effects in HAECs via Fc γ RI (CD64) and Fc γ RII (CD32) for stimulation and inhibition of IL-8 and prostacyclin release respectively [30]. We also showed that piceatannol (syk kinase inhibitor) could reverse these effects suggesting the involvement of Fc γ RIIa mediated signaling [30]. Thus, in the current study, we explored the role of specific Fc γ R type by treatment of cells with 10 μ g/ml of specific Abs separately for 1 hour prior to CRP treatment. We also tested whether the inhibitory effects of CRP can be blocked using piceatannol (50 μ M for 1 hour).

Statistical analysis

All experiments were performed at least three times in duplicate. Experimental results are presented as the means $\pm$ SEM. Paired *t* tests were used to compute differences in the variables, and the level of significance was set at *P*<0.05.

Results

Incubation of HAECs with CRP (0 to 50 μ g/mL) for 12 hours resulted in significant inhibition of eNOS mRNA as reported by us previously [6] (Fig 1a). Furthermore, a significant decrease in citrulline production as well as cGMP release (*P*<0.05 at doses $\geq$ 25 μ g/mL; Figure 1 b & c respectively) was also observed.

The coupling/uncoupling of eNOS activity is reported to be regulated by BH4 availability [23,24]. CRP treatment resulted in a significant decrease in intracellular BH4 levels (reduced form) as compared to control cells (Fig 2a). Furthermore, sepiapterin (a BH4 precursor) treatment prevented the decrease in BH4 levels (Fig 2 b) as well as reversal of eNOS inhibition (Fig 2 c & d).

GTPCH1 is the first enzyme in the de novo biosynthesis of BH4. CRP treatment of HAECs resulted in significant inhibition of GTPCH1 mRNA expression (Fig 3a) and activity (Fig 3b).

Thus, our results demonstrate that CRP downregulates GTPCH1 gene expression and its enzymatic activity resulting in decreased BH4 levels.

GTPCH1 activity is regulated by cAMP mediated pathway [26]. In the present study, Br-cAMP or Db-cAMP pretreatment to cells led to significant reversal of CRP-mediated inhibition of GTPCH1 activity (Fig 3c). Furthermore, eNOS dysfunction has been reported to be attenuated via cAMP/PKA pathway activation [31,32]. The pretreatment of HAECs with cAMP analogues led to significant reversal of CRP-mediated eNOS inhibition (Fig 3d and e) in the present study.

We also investigated whether eNOS dimerization was altered by CRP treatment. The ratio of eNOS dimers to monomers was significantly lower ($n=5$, $p<0.02$) in CRP treated cells as compared to control supporting eNOS uncoupling (Fig 4).

Since increased superoxide production could result in a decrease in eNOS activity, we explored the specific enzymatic source for CRP-mediated superoxide generation in HAECs. As shown in Fig 5a, there was significant inhibition of CRP-induced superoxide production by pharmacologic inhibition of NADPH oxidase as well as siRNA to p47 phox and not control siRNA. We also demonstrate CRP-mediated upregulation of p22 phox protein (Fig 5b) as well as significant inhibition of superoxide release by using siRNA to p22 phox (Fig 5c). Furthermore, the pretreatment with PKC inhibitors resulted in attenuation of CRP-mediated superoxide release (Fig 5d). As shown in Fig 5e, CRP treated cells demonstrated increased DHE fluorescence which was abrogated by siRNA to p47 and p22 phox. However, XO inhibition had no effect on CRP-mediated superoxide generation in HAECs (Control: 0.06 ± 0.02 ; CRP: 0.13 ± 0.03 ; CRP + Allopurinol $\{10\mu\text{M}\}$: 0.11 ± 0.04 [nmol/min/mg protein; $p<0.05$, control vs. CRP and $p>0.05$, CRP vs. CRP + Allopurinol]). Additionally, inhibition of mitochondrial respiratory enzymes had no effect on CRP-mediated superoxide generation (data not shown). Also, pretreatment with L-NAME significantly reversed CRP mediated superoxide production (Control: 0.04 ± 0.01 ; CRP: 0.15 ± 0.03 ; CRP+L-NAME: 0.06 ± 0.03 [$p<0.05$ for control vs. CRP and for CRP vs. CRP+NAME]). In addition, sepiapterin pretreatment of HAECs 1 hr prior to CRP exposure led to significant inhibition of superoxide production (C 0.09 ± 0.02 , CRP: 0.21 ± 0.06 , Sepiapterin + CRP: 0.13 ± 0.05 ; $p<0.05$ - C vs. CRP and for CRP vs. sepiapterin + CRP). We demonstrate in the present study that the use of NADPH oxidase inhibitors as well as siRNA to p47 phox and not control siRNA reversed CRP-mediated inhibition of eNOS (Fig 5e). However, use of inhibitors to xanthine oxidase, arginase and mitochondrial respiratory chain enzymes did not result in attenuation of CRP-mediated eNOS inhibition (Table 1). Thus, NADPH oxidase seems to be involved in CRP-mediated eNOS inhibition.

To determine whether CRP treatment results in alteration of VEGF- induced phosphorylation at Ser1177 and dephosphorylation at Thr495, we performed immunoprecipitation of cell lysates of CRP treated and control cells with eNOS Ab. An increase in Ser1177 phosphorylation and a decrease in Thr 495 phosphorylation occurred in control cells but not in CRP-treated cells (Fig 6a & b [i and ii]). Under these conditions, eNOS abundance was found to be same in both control and CRP treated cells (Fig 6a) at the time point tested in the present study.

CRP-mediated effects in HAECs have been reportedly shown to occur via $\text{Fc } \gamma \text{ Rs}$ [30]. The use of antibodies to CD32 and CD64, but not CD16 or isotype control, significantly reversed CRP mediated eNOS downregulation (Fig 7a and b) as well as GTPCH1 activity (Fig 7c). Furthermore, we also show that piceatannol, syk kinase inhibitor, could significantly reverse CRP-mediated effects on both of the above reported events i.e GTPCH1 activity (Fig 7c) as well as eNOS inhibition (cGMP [fmol/mg protein]: C -258 ± 45 , CRP- 114 ± 39 , CRP +Piceatannol- 229 ± 59 , $p<0.05$ C vs CRP and for CRP vs CRP+Piceatannol; citrulline release

[pmol/mg protein]:C-423 $\pm$ 65, CRP-158 $\pm$ 78, CRP+Piceatannol-387 $\pm$ 78, $p < 0.05$ C vs CRP and for CRP vs CRP+Piceatannol).

Discussion

CRP levels are correlated with increased risk for CVD [1,2] and with endothelial dysfunction [6,7] related to reduced NO bioavailability. Previously, we and others [6,7] have shown that CRP causes downregulation of eNOS in human ECs. Additionally, three groups have independently shown in vivo that human CRP Tg mice exhibit decreased eNOS activity [8-10]. Importantly, Grad et al [9] reported significantly decreased mRNA and protein expression of eNOS following vascular injury in human CRP Tg mice. CRP has also been shown to induce hypertension in CF1 Tg mice expressing rabbit CRP (CF1-CRP) attributable to CRP-induced decline in bioavailable NO [33]. However, the molecular mechanisms for the inhibition of eNOS by CRP have not been elucidated.

In the present study, CRP exerted its inhibitory effect at concentrations of 12.5- 50 ug/ml. CRP levels of 25 and 50 ug/ml have been reported in patients with acute coronary syndromes [34, 35]. Thus, the levels of CRP used in this study can clearly be attained in patients.

Endothelial dysfunction has been linked to reduced BH4 availability and uncoupling of eNOS [15,36,37]. Various clinical studies have also demonstrated that administration of BH4 improves endothelial dysfunction in conditions characterized by reduced availability of NO and increased production of reactive oxygen species, such as hypercholesterolemia [38], coronary artery disease [24] and smoking [39]. BH4 is synthesized through 2 distinct pathways [16]. One is the de novo synthetic pathway in which the precursor GTP is catalyzed by GTPCH1, the first enzyme in this biosynthetic pathway, to dihydroneopterin triphosphate, whereby BH4 is then generated by further steps involving 6-pyruvyltetrahydropterin synthase (PTPS) and sepiapterin reductase. Recently, reduced expression and activity of GTPCH1 has been shown in coronary endothelial cells of diabetic rats and aortae of insulin-resistant rats, resulting in diminished BH4 levels [40,41]. In this context, Cai et al [40] have reported that GTPCH1 gene transfer augments intracellular BH4 as well as eNOS activity in human endothelial cells. Based on these findings, GTPCH1 is reported to be a potential and rational target to augment endothelial BH4 in reversing eNOS activity in endothelial dysfunctional states. Recently, Takaya et al [42] showed that augmenting BH4 levels in the endothelium by GTPCH1 overexpression reduces atherosclerosis in apoE KO/eNOS Tg mice and is associated with a reduction in O₂⁻ from uncoupled eNOS. In the present study, we focused on the effects of CRP on intracellular BH4 levels. We demonstrate that CRP significantly decreased BH4 levels. GTPCH1 is the first enzyme in BH4 biosynthetic pathway. We show for the first time that CRP inhibits GTPCH1 enzyme activity explaining the deficiency of BH4 induced by CRP. Importantly, supplementation of ECs with sepiapterin, BH4 precursor, in the present study resulted not only in normalization of CRP-mediated decrease in BH4 levels but also reversal of superoxide production as well as eNOS inhibition. Thus, we conclude that BH4 supplementation may restore eNOS activity in CRP treated HAECs.

Furthermore, GTPCH1 enzyme has been reported to be regulated by cAMP- mediated pathway [26]. In this context, cell permeable analogue of cAMP, 8 Br-cAMP, which activates protein kinase A has been shown not only to result in restoration of GTPCH1 activity [26], but also attenuation of eNOS dysfunction [31,32]. Importantly, Hashimoto et al [43] reported the activation of eNOS by cilostazol via a cAMP/PKA and PI3K/Akt dependent mechanism. We have reported [30] previously that CRP treatment significantly inhibits cAMP levels in human-monocytes derived macrophages resulting in their pro-inflammatory phenotype. Notably, we reveal here significant reversal of CRP-mediated GTPCH1 activity as well as eNOS downregulation by using cAMP analogues.

Dimerization is an absolute requirement for eNOS catalytic activity [22,40]. Structural studies suggest a role for BH4 in NOS dimerization based on purified recombinant proteins in reconstituted cell-free systems [22]. In the current study, we found that CRP decreased the eNOS dimer/monomer ratio further supporting eNOS uncoupling.

A growing body of evidence suggests a pivotal role of the NADPH oxidase in vascular oxidant stress resulting in eNOS uncoupling [17]. Our group has previously reported that CRP induces the production of superoxide anion in HAECs [5]. The different sources of ROS in vascular cells are NADPH oxidase, mitochondrial respiratory chain enzymes, xanthine oxidase and uncoupled eNOS [17]. Qamirani et al [18] reported that CRP treatment to coronary arterioles in-vitro inhibits endothelium dependent NO-mediated dilatation by activating p38 kinase and NADPH oxidase. Furthermore, Kobayashi et al [19] reported the colocalization of CRP with p22 phox subunit of NADPH oxidase in VSMCs. These authors also reported that CRP directly enhanced the expression of NADPH oxidase p22 phox protein as well as the generation of ROS in cultured human coronary artery SMCs. All these studies suggest that CRP induces oxidative stress via NADPH oxidase. Hence, we examined the involvement of different sources of ROS in CRP-induced superoxide production in HAECs. Neither the inhibition of mitochondrial respiratory chain enzymes nor xanthine oxidase attenuated CRP-mediated O_2^- production. However, NADPH oxidase inhibitors as well as siRNA to p47 phox as well as p22 phox attenuated CRP-induced O_2^- production. Importantly, the PKC pathway is involved in activation of NADPH oxidase via phosphorylation of p47 phox [17]. Hence, we further explored the role of PKC pathway in CRP mediated superoxide release. PKC inhibition significantly inhibited CRP-mediated superoxide release. Overall, our findings suggest that the PKC pathway is involved in CRP-induced activation of NADPH oxidase resulting in superoxide production. Furthermore, CRP-mediated eNOS downregulation was attenuated via inhibition of NADPH oxidase, but not XO or mitochondrial respiratory chain enzymes. Overall, it implies that CRP promotes ROS via NADPH oxidase activation resulting in uncoupling of eNOS. Our results are supported by recently published report by Xu et al [44] demonstrating that NADPH oxidase plays a very important role in eNOS uncoupling mediated by hypochlorous acid (HOCl), the major oxidant of leukocyte-derived myeloperoxidase (MPO). Additionally, L-NAME (a non selective NOS inhibitor) resulted in significant inhibition of CRP-induced superoxide production further supporting eNOS uncoupling [28].

Also, emerging evidence demonstrates that arginase competes with NOS for the substrate L-arginine. Arginase is reported to reciprocally regulate NOS [29] by modulating L-arginine bioavailability. Arginase upregulation is reported to contribute to impaired NO signaling as a result of aging [45] as well as following treatment of ECs to Ox-LDL [29] and is suggested to be a therapeutic target. However, arginase inhibition failed to affect CRP-mediated eNOS inhibition, excluding arginase upregulation in the present study.

The activity of eNOS can be regulated by changes in its phosphorylation at serine residue (Ser1177) in the reductase domain and threonine residue (Thr495) in the CaM binding domain. Phosphorylation at Ser1177 and dephosphorylation at Thr495 of eNOS leads to enhanced activity of the enzyme, and thus augmented NO production [31,46]. In the present study, we show that CRP causes potent antagonism of eNOS activation by VEGF resulting in decrease in eNOS phosphorylation at Ser1177 and increased phosphorylation at Thr 495. Our results are supported by a previously published study [8] that showed CRP-mediated eNOS inhibition is attributable to blunted eNOS phosphorylation at Ser1179 in response to activation with diverse agonists (VEGF, HDL and insulin) in bovine aortic ECs.

The known actions of CRP are mediated by FcγRs, with FcγRI (CD64) and II (CD32) but not III (CD16), acting as the principal receptor pathway in mediating CRP's effects in HAECs. Our group has previously shown that CRP-mediated increase and decrease in IL-8 and

prostacyclin respectively occurs via Fc γ receptors I and II [30]. Piceatannol (syk kinase inhibitor) could also reverse these effects suggesting the involvement of Fc γ RIIa mediated signaling [30]. Recently, Mineo et al [8] reported CRP-mediated eNOS downregulation to be via Fc γ RIIb in COS-7 cells transfected with Fc γ RIIb cDNA. Furthermore, these authors also reported CRP-mediated blunting of acetylcholine-induced increase in carotid artery vascular conductance in Fc γ IIb $^{+/+}$ mice. However, these authors failed to show the mRNA expression of Fc γ RIIa in HAECs. In the present study, we report the involvement of both CD32 and CD64 in mediating CRP's effects since CRP mediated eNOS downregulation as well as GTPCH1 activity was significantly inhibited by pretreatment with the Abs to CD32 and CD64. Also, we tested whether the reported biological effects of CRP can be blocked using piceatannol because signaling through Fc γ receptors requires activation of Syk kinase [47]. The Syk inhibitor was able to significantly reverse the inhibitory effect of CRP on the activity of eNOS as well as GTPCH1. Thus, we again demonstrate that CRP mediates its multiple pro-inflammatory effects including eNOS inhibition via uptake through the Fc γ Rs in HAECs.

Since the major focus of our study was to elucidate the molecular mechanism of eNOS inhibition, we confirmed our findings in HAECs in vitro. However, studies directed at confirming our findings in-vivo in a rat model are underway.

In the present study, there are three major new findings as depicted in schema (Fig 8): CRP decreases BH $_4$ levels due to inhibition of GTPCH1 resulting in uncoupling of eNOS; CRP causes the activation of NADPH oxidase resulting in eNOS uncoupling by altering eNOS dimerization; CRP treatment blunts agonist (VEGF) induced eNOS phosphorylation at Ser1177 and dephosphorylation at Thr495. Thus, given the importance of CRP-induced pro-oxidative effects and resultant eNOS inhibition, CRP appears to be a key molecule to accentuate endothelial dysfunction. To conclude, strategies aimed at decreasing CRP [48] may prove to be beneficial in preventing endothelial dysfunction and reducing atherosclerosis related events.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Ridker PM, Cook N. Clinical usefulness of very high and very low levels of CRP across the full range of Framingham Risk Scores. *Circulation* 2004;109:1955–9. [PubMed: 15051634]
2. Ridker PM. Clinical application of C-reactive protein for cardiovascular disease detection and prevention. *Circulation* 2003;107:363–9. [PubMed: 12551853]
3. Jialal I, Devaraj S, Venugopal SK. C-reactive protein: risk marker or mediator in atherothrombosis? *Hypertension* 2004;44:6–11. [PubMed: 15148294]
4. Ballou SP, Lozanski G. Induction of inflammatory cytokine release from cultured human monocytes by CRP. *Cytokine* 1992;4:361–8. [PubMed: 1420997]
5. Verma S, Devaraj S, Jialal I. Is C-reactive protein an innocent bystander or proatherogenic culprit? C-reactive protein promotes atherothrombosis. *Circulation* 2006;113:2135–50. [PubMed: 16671184] Review
6. Venugopal SK, Devaraj S, Yuhanna I, Shaul P, Jialal I. Demonstration that CRP decreases eNOS expression and bioactivity in human aortic endothelial cells. *Circulation* 2002;106:1439–41. [PubMed: 12234944]

7. Verma S, Wang CH, Li SH, Dumont AS, Fedak PW, Badiwala MV, Dhillon B, Weisel RD, Li RK, Mickle DA, Stewart DJ. A self-fulfilling prophecy: CRP attenuates nitric Oxide production and inhibits angiogenesis. *Circulation* 2002;106:913–9. [PubMed: 12186793]
8. Mineo C, Gormley AK, Yuhanna IS, Osborne-Lawrence S, Gibson LL, Hahner L, Shohet RV, Black S, Salmon JE, Samols D, Karp DR, Thomas GD, Shaul PW. Fc gamma RIIB mediates CRP inhibition of endothelial NO synthase. *Circ Res* 2005;97:1124–31. [PubMed: 16269657]
9. Grad E, Golomb M, Mor Yosef I, Koroukhov N, Lotan C, Edelman ER, Danenberg HD. Transgenic expression of human CRP suppresses endothelial nitric oxide synthase expression and bioactivity following vascular injury. *Am J Physiol Heart Circ Physiol* 2007;293:H489–95. [PubMed: 17369452]
10. Schwartz R, Osborne-Lawrence S, Hahner L, Gibson LL, Gormley AK, Vongpatanasin W, Zhu W, Word RA, Seetharam D, Black S, Samols D, Mineo C, Shaul PW. CRP downregulates endothelial NO synthase and attenuates reendothelialization in vivo in mice. *Circ Res* 2007;100:1452–9. [PubMed: 17446434]
11. Fichtlscherer S, Rosenberger G, Walter DH, Breuer S, Dimmeler S, Zeiheret AM. Elevated CRP and impaired endothelial reactivity in patients with CAD. *Circulation* 2000;102:1000–6. [PubMed: 10961964]
12. Cleland SJ, Sattar N, Petrie JR, Forouhi NG, Elliott HL, Connell JM. Endothelial dysfunction as a possible link between C-reactive protein levels and cardiovascular disease. *Clin Sci* 2000;98:531–5. [PubMed: 10781383]
13. Tomai F, Crea F, Gaspardone A, Versaci F, Ghini AS, Chiariello L, Gioffre PA. Unstable angina and elevated c-reactive protein levels predict enhanced vasoreactivity of the culprit lesion. *Circulation* 2001;104:1471–6. [PubMed: 11571238]
14. Whitsett J, Martasek P, Zhao H, Schauer DW, Hatakeyama K, Kalyanaraman B, Vasquez-Vivar J. Endothelial cell superoxide anion radical generation is not dependent on eNOS- serine 1179 phosphorylation and eNOS dimer/monomer distribution. *Free Radic Biol Med* 2006;40:2056–68. [PubMed: 16716906]
15. Vasquez-Vivar J, Kalyanaraman B, Martasek P, Hogg N, Masters BS, Karoui H, Tordo P, Pritchard KA Jr. Superoxide generation by endothelial nitric oxide synthase: the influence of cofactors. *Proc Natl Acad Sci U S A* 1998;95:9220–5. [PubMed: 9689061]
16. Thony B, Auerbach G, Blau N. Tetrahydrobiopterin biosynthesis, regeneration and functions. *Biochem J* 2000;347:1–16. [PubMed: 10727395]
17. Griendling KK, Sorescu D, Ushio-Fukai M. NAD(P)H oxidase: role in cardiovascular biology and disease. *Circ Res* 2000;86:494–501. [PubMed: 10720409]
18. Qamirani E, Ren Y, Kuo L, Hein TW. CRP inhibits endothelium-dependent NO-mediated dilation in coronary arterioles by activating p38 kinase and NAD(P)H oxidase. *Arterioscler Thromb Vasc Biol* 2005;25:995–1001. [PubMed: 15718491]
19. Kobayashi S, Inoue N, Ohashi Y, Terashima M, Matsui K, Mori T, Fujita H, Awano K, Kobayashi K, Azumi H, Ejiri J, Hirata K, Kawashima S, Hayashi Y, Yokozaki H, Itoh H, Yokoyama M. Interaction of oxidative stress and inflammatory response in coronary plaque instability: important role of CRP. *Arterioscler Thromb Vasc Biol* 2003;23:1398–1404. [PubMed: 12805076]
20. Singh U, Devaraj S, Dasu MR, Ciobanu D, Reusch J, Jialal I. C-reactive protein decreases interleukin-10 secretion in activated human monocyte-derived macrophages via inhibition of cyclic AMP production. *Arterioscler Thromb Vasc Biol* 2006;26:2469–75. [PubMed: 16917108]
21. Dasu MR, Devaraj S, Du Clos TW, Jialal I. Biological effects of CRP are not due to endotoxin contamination: Evidence from toll like receptor 4 knock-down human aortic endothelial cells. *J Lipid Res* 2006;48:509–12. [PubMed: 17158793]
22. Klatt P, Schmidt K, Lehner D, Glatter O, Bachinger HP, Mayer B. Structural analysis of porcine brain nitric oxide synthase reveals a role for tetrahydrobiopterin and L-arginine in the formation of an SDS-resistant dimer. *EMBO J* 1995 Aug 1;14(15):3687–95. [PubMed: 7543842]
23. Vasquez-Vivar J, Kalyanaraman B, Martasek P, Hogg N, Masters BS, Karoui H, Tordo P, Pritchard KA Jr. Superoxide generation by endothelial nitric oxide synthase: the influence of cofactors. *Proc Natl Acad Sci U S A* 1998;95:9220–5. [PubMed: 9689061]

24. Maier W, Cosentino F, Lutolf R, Fleisch M, Seiler C, Hess OM, Meier B, Lüscher TF. Tetrahydrobiopterin improves endothelial function in patients with coronary artery disease. *J Cardiovasc Pharmacol* 2000;35:173–8. [PubMed: 10672847]
25. Ogiwara S, Kiuchi K, Nagatsu T, Teradaira R, Nagatsu I, Fujita K, Sugimoto T. Highly sensitive, specific enzyme-linked immunosorbent assay of neopterin and biopterin in biological samples. *Clin Chem* 1992;38:1954–8. [PubMed: 1394977]
26. Hirayama K, Shimoji M, Swick L, Meyer A, Kapatos G. Characterization of GTP cyclohydrolase I gene expression in the human neuroblastoma SKN-BE(2)M17: enhanced transcription in response to cAMP is conferred by the proximal promoter. *J Neurochem* 2001;79:576–87. [PubMed: 11701761]
27. Venugopal SK, Devaraj S, Yang T, Jialal I. Alpha-tocopherol decreases superoxide anion release in human monocytes under hyperglycemic conditions via inhibition of protein kinase C- α . *Diabetes* 2002;51:3049–54. [PubMed: 12351446]
28. Pritchard KA Jr, Groszek L, Smalley DM, Sessa WC, Wu M, Villalon P, Wolin MS, Stemerman MB. Native LDL increases endothelial cell NOS generation of superoxide anion. *Circ Res* 1995;77:510–8. [PubMed: 7543827]
29. Ryoo S, Lemmon CA, Soucy KG, Gupta G, White AR, Nyhan D, Shoukas A, Romer LH, Berkowitz DE. Oxidized LDL-dependent endothelial arginase II activation contributes to impaired nitric oxide signaling. *Circ Res* 2006;99:951–60. [PubMed: 17008605]
30. Devaraj S, Du Clos TW, Jialal I. Binding and internalization of CRP by Fc γ receptors on human aortic endothelial cells mediates biological effects. *Arterioscler Thromb Vasc Biol* 2005;25:1359–63.
31. Shah DI, Singh M. Activation of protein kinase A improves vascular endothelial dysfunction. *Endothelium* 2006;13:267–77. [PubMed: 16990183]
32. Shah DI, Singh M. Possible role of exogenous cAMP to improve vascular endothelial dysfunction in hypertensive rats. *Fundam Clin Pharmacol* 2006;20:595–604. [PubMed: 17109653]
33. Vongpatanasin W, Thomas GD, Schwartz R, Cassis LA, Osborne-Lawrence S, Hahner L, Gibson LL, Black S, Samols D, Shaul PW. CRP causes downregulation of vascular angiotensin subtype 2 receptors and systolic hypertension in mice. *Circulation* 2007;115:1020–8. [PubMed: 17283257]
34. de Lemos JA, Blazing MA, Wiviott SD, Lewis EF, Fox KA, White HD, Rouleau JL, Pedersen TR, Gardner LH, Mukherjee R, Ramsey KE, Palmisano J, Bilheimer DW, Pfeffer MA, Califf RM, Braunwald E. A to Z Investigators. Early intensive vs a delayed conservative simvastatin strategy in patients with acute coronary syndromes: phase Z of the A to Z trial. *JAMA* 2004;292:1307–16. [PubMed: 15337732]
35. Morrow DA, Rifai N, Antman EM, Weiner DL, McCabe CH, Cannon CP, Braunwald E. C-reactive protein is a potent predictor of mortality independently of and in combination with troponin T in acute coronary syndromes: a TIMI 11A substudy. *Thrombolysis in Myocardial Infarction. J Am Coll Cardiol* 1998;31:1460–5. [PubMed: 9626820]
36. Shinozaki K, Nishio Y, Okamura T, Yoshida Y, Maegawa H, Kojima H, Masada M, Toda N, Kikkawa R, Kashiwagi A. Oral administration of tetrahydrobiopterin prevents endothelial dysfunction and vascular oxidative stress in the aortas of insulin-resistant rats. *Circ Res* 2000;87:566–73. [PubMed: 11009561]
37. Topal G, Brunet A, Millanvaye E, Boucher JL, Rendu F, Devynck MA, David-Dufilho M. Homocysteine induces oxidative stress by uncoupling of NO synthase activity through reduction of tetrahydrobiopterin. *Free Radic Biol Med* 2004;36:1532–41. [PubMed: 15182855]
38. Stroes E, Kastelein J, Cosentino F, Erkelens DW, Wever R, Koomans HA, Lüscher TF, Rabelink TJ. Tetrahydrobiopterin restores endothelial function in hypercholesterolemia. *J Clin Invest* 1997;99:41–6. [PubMed: 9011574]
39. Heitzer T, Brockhoff C, Mayer B, Warnholtz A, Mollnau H, Henne S, Meinertz T, Münzel T. Tetrahydrobiopterin improves endothelium dependent vasodilation in chronic smokers: evidence for a dysfunctional nitric oxide synthase. *Circ Res* 2000;86:e36–41. [PubMed: 10666424]
40. Cai S, Khoo J, Channon KM. Augmented BH4 by gene transfer restores nitric oxide synthase function in hyperglycemic human endothelial cells. *Cardiovasc Res* 2005;65:823–31. [PubMed: 15721862]
41. Cai S, Alp NJ, McDonald D, Smith I, Kay J, Canevari L, Heales S, Channon KM. GTP cyclohydrolase I gene transfer augments intracellular tetrahydrobiopterin in human endothelial cells: effects on nitric

- oxide synthase activity, protein levels and dimerisation. *Cardiovasc Res* 2002;55:838–49. [PubMed: 12176133]
42. Takaya T, Hirata K, Yamashita T, Shinohara M, Sasaki N, Inoue N, Yada T, Goto M, Fukatsu A, Hayashi T, Alp NJ, Channon KM, Yokoyama M, Kawashima S. A specific role for eNOS-derived reactive oxygen species in atherosclerosis progression. *Arterioscler Thromb Vasc Biol* 2007;27:1632–7. [PubMed: 17463333]
 43. Hashimoto A, Miyakoda G, Hirose Y, Mori T. Activation of endothelial nitric oxide synthase by cilostazol via a cAMP/protein kinase A- and phosphatidylinositol 3-kinase/Akt-dependent mechanism. *Atherosclerosis* 2006;189:350–7. [PubMed: 16545819]
 44. Xu J, Xie Z, Reece R, Pimental D, Zou MH. Uncoupling of endothelial nitric oxidase synthase by hypochlorous acid: role of NAD(P)H oxidase-derived superoxide and peroxynitrite. *Arterioscler Thromb Vasc Biol* 2006;26:2688–95. [PubMed: 17023679]
 45. Berkowitz DE, White R, Li D, Minhas KM, Cernetch A, Kim S, Burke S, Shoukas AA, Nyhan D, Champion HC, Hare JM. Arginase reciprocally regulates nitric oxide synthase activity and contributes to endothelial dysfunction in aging blood vessels. *Circulation* 2003;108:2000–6. [PubMed: 14517171]
 46. Michell BJ, Chen Zp, Tiganis T, Stapleton D, Katsis F, Power DA, Sim AT, Kemp BE. Coordinated control of endothelial nitric-oxide synthase phosphorylation by protein kinase C and the cAMP-dependent protein kinase. *J Biol Chem* 2001;276:17625–8. [PubMed: 11292821]
 47. Chi M, Tridandapani S, Zhong W, Coggeshall KM, Mortensen RF. C-reactive protein induces signaling through FcγRIIIa on HL-60 granulocytes. *J Immunol* 2002;168:1413–8. [PubMed: 11801683]
 48. Pepys MB, Hirschfield GM, Tennent GA, Gallimore JR, Kahan MC, Bellotti V, Hawkins PN, Ley SV, Aquilina JA, Robinson CV, Sharif I, Gray GA, Sabin CA, Jenvey MC, Kolstoe SE, Thompson D, Wood SP. Targeting CRP for the treatment of CVD. *Nature* 2006;440:1217–21. [PubMed: 16642000]

Fig 1.

Effect of CRP on eNOS mRNA expression (a), NOS activity (b), and cGMP release (c). HAECs were incubated with CRP (0, 25 and 50 $\mu\text{g/ml}$) for 12 h. RT-PCR (a) for eNOS mRNA or GAPDH mRNA (as loading control) was performed as described under Methods. 1-C, 2-CRP 25 $\mu\text{g/ml}$, 3- CRP50 $\mu\text{g/ml}$. The determination of NOS activity (b) and cGMP levels (c) was done as described under Methods.* $p < 0.05$ as compared to control. Data are mean $\pm$ SEM of 5-7 experiments in duplicate.

Fig 2.

a&b) Effect of CRP and supplementation with sepiapterin on BH₄ levels in HAECs. BH₄ measurement was done by HPLC as described in methods section. *p<0.05 as compared to control. The results are mean $\pm$ SEM of 5 different experiments done in duplicate. **c & d)** Effect of supplementation with sepiapterin on CRP-mediated eNOS inhibition as measured by (c) eNOS activity and (d) cGMP release. The results are mean $\pm$ SEM of 4 different experiments done in duplicate. *p<0.05 as compared to control, # p<0.05 as compared to CRP.

(a)

(b)

Fig 3.

a & b) Effect of CRP on GTPCH1 mRNA expression (**a**) and activity (**b**) in HAECs. The cells were treated with CRP (0, 25 and 50 μ g/ml) for 12 hrs. RNA was isolated for RT-PCR for GTPCH1 mRNA or GAPDH mRNA (as loading control) as detailed in methods. 1-C, 2-CRP 25 μ g/ml, 3- CRP50 μ g/ml. GTPCH1 enzyme activity (Neopterin generated) was assayed as detailed in methods. The results are mean $\pm$ SD of 3 different experiments done in duplicate and are presented as pmoles/min/mg protein. * $p < 0.05$ as compared to control. **c)** Role of cAMP-mediated pathway in GTPCH1 inhibition and eNOS downregulation (**d and e**) by CRP. Control, CRP (25 μ g/ml), cAMP analogues treated cells [(8-Br-cAMP and Db-cAMP 0.5mM each) 1 hr prior to CRP treatment] were used for the measurement of GTPCH1 activity, eNOS activity and NO bioactivity as described in methods. * $p < 0.05$ as compared to control. # $p < 0.05$ as compared to CRP.

Fig 4.

Effect of CRP on eNOS dimer and monomer forms. HAECs were treated with CRP for 12 hrs as reported in methods. The lysates were run on low temperature SDS-PAGE at 70 V on 6% mini-gels. The proteins were transferred on PVDF membrane and blotted for eNOS forms using rabbit anti-human eNOS Ab (1:1000) and anti-rabbit IgG (1:2000). Actin was used as house keeping gene. The upper panel shows the blot which is representative of 5 different experiments. The lower panel shows the densitometric ratio of eNOS dimer:monomer. * $p < 0.02$ compared to control.

Fig 5.

(a) Role of NADPH oxidase inhibition on CRP-mediated superoxide production. The cells were transfected with siRNA to p47 phox or control siRNA as detailed in methods section. The cells were incubated with DPI (5 μ mol/L) 1 hour prior to CRP treatment (25 ug/ml for 6 hrs). O_2^- production was measured as described in methods. The results are mean $\pm$ SEM of 4 different experiments. * $p < 0.01$ as compared to control, # $p < 0.05$ as compared to CRP, ** $p < 0.05$ as compared to control, ## $p < 0.05$ as compared to CRP. **(b)** The cells were transfected with siRNA to p22 phox/CSiRNA or control siRNA as detailed in methods section. At the end of incubation with CRP treatment, western blotting was done for p22 phox and actin as described in methods. Density ratio of p22phox/actin is provided. * $p < 0.05$ as compared to control. **(c)** Role of p22 phox inhibition on CRP-mediated superoxide production. The cells were transfected with siRNA to p22 phox or control siRNA and superoxide release was measured as detailed in methods section. * $p < 0.01$ as compared to control, # $p < 0.05$ as compared to CRP. The results are mean $\pm$ SEM of 4 different experiments. **(d)** Role of PKC in CRP-mediated superoxide release. The cells were pretreated with PKC inhibitors prior to CRP treatment and superoxide release was measured as detailed in methods. * $p < 0.01$ as compared to control, # $p < 0.05$ as compared to CRP. The results are mean $\pm$ SEM of 3 different experiments. **(e)** Effect of p47 and p22 phox siRNA on CRP-mediated superoxide release as measured by DHE fluorescence as detailed in methods- representative figure of $n=3$ experiments. **(f)** Role of NADPH oxidase inhibition on CRP- mediated eNOS inhibition. The cells were incubated with NADPH-oxidase inhibitors (apocynin [0.6 mM] and DPI [5 μ M]) 1 hour prior to CRP treatment (25 μ g/ml for 12 hrs). The cells were transfected with siRNA to p47 phox or control siRNA as detailed in methods section. At the end of incubation with CRP treatment, eNOS activity and cGMP release were measured as described in methods. The results are mean $\pm$ SEM of 4 different experiments done in duplicate. * $p < 0.05$ as compared to control, # $p < 0.05$ as compared to CRP.

(a)

Immunoblots for Ser-1177/Thr-495 eNOS, hsp90 and eNOS after IP with eNOS antibody

Fig 6.

a) Western blots showing time course of VEGF (100 ng/ml for 0-15 min)-induced phosphorylation of Ser1177 and dephosphorylation of Thr495 in control and CRP treated cells. The cell lysates were immunoprecipitated for eNOS and subjected to SDS-PAGE and blotted using specific Abs as described in methods section. bi & ii) Phosphorylation was quantified densitometrically as the ratio of phospho-eNOS/eNOS for blot in Fig 6a. Results are presented as mean $\pm$ SEM of 3 different experiments. * $p < 0.05$ as compared to control.

Fig 7. Effect of Fc γ R neutralizing antibodies on CRP-mediated eNOS inhibition. HAECs were incubated with respective antibodies for 1 hour prior to CRP treatment as described in methods

followed by the measurement of (a) NOS activity and (b) cGMP. * $p < 0.05$ compared with control, # $p < 0.05$ compared with CRP. The results are mean $\pm$ SEM of 3 experiments in duplicate. (c) Effect of Fc γ R neutralizing antibodies and syk kinase inhibitor on CRP-mediated GTPCH1 activity inhibition. * $p < 0.05$ compared with control, # $p < 0.05$ compared with CRP. The results are mean $\pm$ SEM of 3 experiments in duplicate.

Schema

Fig 8.

Hypothetical schema illustrating the possibility of divergent effects for CRP- mediated eNOS inhibition. CRP uncouples eNOS via 2 different ways: stimulation of NADPH oxidase leading to generation of oxidative stress which causes the formation of superoxide rather than NO from eNOS; CRP also inhibits GTPCH1, the rate limiting enzyme for de novo synthesis of BH₄, resulting in decreased BH₄ levels. The uncoupled eNOS (as evidenced by decreased dimer:monomer ratio) results in altered phosphorylation of Ser1177 and Thr495 finally leading to decreased functional activity of eNOS.

Table1

Effect of inhibitors to mitochondrial respiratory chain enzymes, xanthine oxidase and arginase on CRP -mediated eNOS inhibition

	cGMP (fmol/mg protein)	Citrulline (pmol/mg protein)
Control	311±45	487±102
CRP (25µg/ml)	140±34 [*]	242±69 [*]
CRP+ TTFA	108±56 [*]	246±45 [*]
CRP+ HOCA	125±34 [*]	264±89 [*]
CRP+ AOAC	132±39 [*]	286±67 [*]
CRP+ Rotenone	155±21 [*]	242±46 [*]
CRP+ Allopurinol	147±23 [*]	268±76 [*]
CRP+ BEC	155±34 [*]	264±56 [*]

All values are mean ± SD from 3 experiments done in duplicate.

* p<0.05 as compared to control cells

Abbreviations: Theonyl-trifluoroacetone, TTFA; alpha-cyano-4-hydroxycinnamic acid, (HOCA); aminooxyacetic acid, AOAC; BEC, (S)-(2-boronoethyl)-L-cysteine HCl.