

Transgenic expression of human C-reactive protein suppresses endothelial nitric oxide synthase expression and bioactivity after vascular injury

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Grad E, Golomb M, Mor-Yosef I, Koroukhov N, Lotan C, Edelman ER, Danenberg HD. Transgenic expression of human C-reactive protein suppresses endothelial nitric oxide synthase expression and bioactivity after vascular injury. *Am J Physiol Heart Circ Physiol* 293: H489–H495, 2007. First published March 16, 2007; doi:10.1152/ajpheart.01418.2006.—C-reactive protein (CRP) is a risk marker and a potential modulator of vascular disease. Whether CRP modulates nitric oxide (NO) synthase (NOS) activity and NO metabolism remains unclear. We studied the effect of CRP on NO metabolism in transgenic mice that express human CRP (CRP_{tg}). CRP_{tg} and wild-type mice were subjected to controlled femoral artery wire injury. CRP serum levels at baseline and 6 and 24 h after injury were 12.4 ± 9 , 18.6 ± 6.9 , and 58.4 ± 13 mg/l, respectively, in CRP_{tg} mice but were undetectable at all time points in wild-type mice. Endothelial NOS protein and mRNA expression were significantly suppressed in the injured arteries of CRP_{tg} mice ($n = 5$, $P < 0.05$). A similar reduction in eNOS expression was observed in the distant lung and heart. NO release after injury was significantly lower in CRP_{tg} mice, as measured by nitrate and nitrite breakdown products, with a concomitant suppression of cGMP NO signaling after injury. Endothelial NOS and NO expression after vascular injury are locally and systemically suppressed in mice that express human CRP. These *in vivo* observations support the hypothesis that CRP modulates NO metabolism and may have implications regarding the mechanisms by which CRP modulates vascular disease.

vascular disease; inflammatory response

C-REACTIVE PROTEIN, an inflammatory acute-phase reactant, is a strong predictor for cardiovascular disease. The predictive value of CRP is independent of other risk markers and common to subjects with and without other “traditional” risk factors for cardiovascular disease (20–22). Numerous reports suggest that CRP is not merely a marker but, also, a modulator that drives direct biological effects on vascular cells. We previously showed accelerated arterial thrombosis in mice that express human CRP (4). CRP increases the expression of intercellular adhesion molecule-1, vascular cell adhesion molecule-1, and E-selectin adhesion molecules in human endothelial cells (15, 38) and induces expression of monocyte chemoattractant protein-1 in endothelial cells (16) and monocytes (10). CRP induces plasminogen activator inhibitor type 1 expression and activity in human aortic endothelial cells (6) via upregulation of NF- κ B activity. CRP-induced suppression of nitric oxide (NO) synthase (NOS) expression and bioactivity in several lines of endothelial cells reduces

endothelial NO production and affects angiogenesis and apoptosis (32, 34, 35). Furthermore, CRP modulates vaso-reactivity in human arterial rings by a non-endothelium-dependent mechanism (25).

Recent studies suggest that these findings, which are based on *in vitro* studies, are artifactual because of contamination of commercial CRP preparations with sodium azide or LPS. When CRP preparations that are dialyzed free of these contaminants are used, several pathobiological effects, including increased intercellular adhesion molecule-1 expression, suppression of von Willebrand factor, and increased monocyte chemoattractant protein-1 and IL-8 secretion (1, 16, 33), are not reproduced (12, 27, 29, 31). Moreover, the suppressive effect of CRP on NOS expression that was lost after dialysis was reinduced by the contaminant sodium azide (29). Other studies done with dialyzed and ascites-derived azide and LPS-free CRP verified a direct procoagulant and proinflammatory activity of CRP (23, 24). The mystery is further complicated by a report of increased NO availability and endothelium-dependent hyporeactivity of vascular rings in response to CRP (3).

NO is a key modulator in the maintenance of vascular homeostasis. NO production can be reduced by endothelial dysfunction along several pathways of vascular injury. To clarify the controversial *in vitro* data that show an association of CRP with NO pathways, we examined the constitutive and postinjury CRP effect on NOS and NO activity *in vivo* in transgenic mice that express human CRP.

MATERIALS AND METHODS

Mice. C57BL/6 congenic CRP_{tg} mice carry a 31-kb *Clal* I fragment of human genomic DNA consisting of the *CRP* gene, 17 kb of 5'-flanking sequence, and 11.3 kb of 3'-flanking sequence (2, 28). The protocol for this study was approved by the Institutional Animal Care and Use Committee. Inasmuch as CRP expression is linked to a testosterone promoter, only male CRP_{tg} mice were used. These CRP_{tg} and age-matched (8–12 wk old) congenic wild-type mice were subjected to bilateral femoral artery wire injury (4). After anesthesia with ketamine (400 μ g/kg) and xylazine (5 mg/kg), mice were placed on a warm (37°C) operating board. A longitudinal groin incision exposed the femoral vessels, and under surgical microscopic visualization (Carl Zeiss) the distal portion of the femoral artery was encircled with a 7-0 nylon suture. A vascular clamp was placed proximally at the level of the inguinal ligament, and an angioplasty guidewire (0.01 in. diameter; Advanced Cardiovascular Systems) was introduced into the arterial lumen through an arteriotomy just distal to the suture. After release of the clamp, the guidewire was advanced to

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the level of the aortic bifurcation and pulled back three times, and the arteriotomy site was ligated. CRPtg ($n = 30$) and matching wild-type congenic ($n = 30$) mice were killed by isoflurane inhalation and mediastinotomy before and 6 and 24 h after injury (10 animals at each time point). Blood was drawn from the left ventricle, and femoral arteries, heart, and lungs were harvested. Five arteries from CRPtg or wild-type mice at each time point (0, 6, and 24 h) were fixed in 10% formalin, and the remainder were snap-frozen in liquid nitrogen. Protein was extracted from heart and lungs of five animals at each time point (0, 6, and 24 h), and mRNA was extracted from arteries and lungs of five animals at each time point (0, 6, and 24 h).

A turbidometry method with a sensitivity of 0.1 mg/l (Roche, Mannheim, Germany) was used to measure serum human CRP levels in all animals.

Quantitative real-time PCR. Total RNA was isolated from frozen lung tissues using TriReagent (Sigma). cDNA was synthesized from 1 μ g of RNA with the use of hexamer primer and avian myeloblastosis virus reverse transcriptase (Boehringer Mannheim, Germany). Quantitative real-time PCR analysis was performed on a sequence detector system (TaqMan ABI Prism 7000, Applied Biosystems, Foster City, CA). cDNA was synthesized from 1 μ g of RNA. Primers and their corresponding fluorescent probes were designed and synthesized by Applied Biosystem's "Assays-by-Design" custom service. Cycling parameters were as follows: 2 min at 50°C for probe and primer activation, 10 min at 95°C for DNA strand denaturation followed by 40 cycles for 15 s each at 95°C for denaturation, and, finally, primer extension for 1 min at 65°C. The sample volume was 20 μ l. Each cDNA sample was tested in triplicate, and mean cycle threshold (C_t) values were reported. For each reaction, a "no-template" sample was included as a negative control. ΔC_t of each sample was calculated as follows: C_t of the reference gene [i.e., L19, which is constitutive and stable during vascular injury (19)] - C_t of the target gene (eNOS). Then the median ΔC_t sample of the wild-type mice at each time point was chosen as the reference for the relative quantification calculation ($2^{-\Delta\Delta C_t}$). Data are expressed as relative quantification (mean \pm SD). Real-time PCR assays for eNOS were performed repeatedly to compare expressions of each congene at different times after injury and to compare the wild-type with the CRPtg response.

RT-PCR. TriReagent (Sigma) was used to isolate total RNA from frozen artery tissues. Hexamer primer and avian myeloblastosis virus reverse transcriptase were used to synthesize cDNA from 0.5 μ g of RNA. For PCR we used the Ready Mix enzyme (Bio-Lab) according to the manufacturer's instructions. The primer set was 5'-CCAGCTGTGTCCAACATGCTG-3' (forward) and 5'-GATGGAAGACAGGAGTTAGG-3' (reverse) for eNOS and 5'-ATGCCAACTCTCGTCAACAG-3' (forward) and 5'-GCGCTTTCGTCGTTTCCTT-3' (reverse) for L19.

Immunohistochemical assay. Arteries were fixed in formalin and embedded in paraffin, and 5- μ m cross sections were used for immunofluorescence studies. Paraffin was removed with standard xylene washes. The slides were boiled for 3 min with 20 mM citrate buffer (pH 6) in a pressure cooker to allow antigen retrieval. To avoid nonspecific binding, the tissue sections were blocked using 3% BSA in 0.1% Tween in PBS for 1 h at room temperature. The sections were incubated overnight with anti-eNOS (BD Transduction Laboratories; 1:100 dilution) at 4°C. Sections incubated without the primary antibody, under otherwise identical conditions, were used as negative control to verify the specificity of the antibody. After they were washed in PBS, the sections were incubated with goat anti-rabbit Cy5 antibody (Jackson ImmunoResearch Lab; 1:200 dilution) for 1 h.

Western blot analysis. Tissues were lysed, homogenized under ultrasound, and boiled for 5 min. After quantification of the protein concentration by the Bradford method, proteins from five animals at each time point were analyzed separately and as a pool. Extracts (20 μ g) were loaded onto 5–15% gradient SDS polyacrylamide gel, and transferred proteins were blotted overnight for eNOS (1:500 dilution;

LAB VISION). eNOS protein expression was normalized to β -actin expression. The bands on the X-ray film were quantified by scanning densitometry (Tina analysis software) and expressed as percentage of wild-type control.

Measurement of NO breakdown products and cGMP concentration. cGMP and nitrite were assayed in lung and heart homogenates. NO production (μ mol/mg protein) was measured with a commercial nitrate/nitrite colorimetric assay kit (Cayman Chemical) according to the manufacturer's instructions. cGMP (pmol/mg protein) was measured by competitive enzyme immunoassay (Cayman Chemical) according to the manufacturer's instructions.

Statistics. Values are means \pm SD. Student's *t*-test and ANOVA with Newman-Keuls post hoc test was used to assess differences between two and more than two groups, respectively. Differences were considered significant at $P < 0.05$.

RESULTS

Baseline serum CRP levels in male CRPtg mice (12.4 ± 9 mg/l) rose 1.5-fold to 18.6 ± 6.9 mg/l at 6 h after vascular injury and 4.7-fold to 58.4 ± 13 mg/l 24 h after vascular injury ($n = 10$, $P < 0.05$). CRP was not detected in serum from wild-type mice before and after injury (not shown). Patchy alopecia was observed in CRPtg mice at 3 mo of age, but not in wild-type mice.

Regulation of arterial NOS by CRP after injury. NOS expression in the femoral artery 1 day after wire injury was measured by semiquantitative RT-PCR. The minute amounts of RNA from a single injured artery did not allow a more quantitative assay of real-time PCR. eNOS mRNA was significantly reduced in CRPtg mice; eNOS expression relative to L19 constitutive expression was almost fivefold greater in CRPtg than in wild-type mice (Fig. 1, A and B; $P < 0.05$).

Immunohistochemical staining for eNOS protein revealed very scarce staining in intact femoral arteries of wild-type and CRPtg mice (Fig. 1, C and D), with minimal endothelial staining 6 h after injury (Fig. 1, E and F), probably because of the almost complete endothelial denudation caused by the wire injury. In arteries harvested 24 h after injury, eNOS was expressed in the endothelium and in subendothelial layers and was significantly lower in CRPtg than in wild-type mice (Fig. 1, G and H).

NOS protein and mRNA in distant noninjured tissues. Reduction of eNOS expression by CRP in lung tissue was studied by Western blot (Fig. 2A). Quantitative analysis demonstrates 36% and 96% reduction in NOS expression in CRPtg mice 6 and 24 h after vascular injury, respectively (Fig. 2A; $n = 5$, $P < 0.05$). Real-time PCR assays for eNOS in the lung showed that constitutive expression of eNOS before injury was similar in wild-type and CRPtg mice. However, eNOS mRNA levels were significantly increased in wild-type mice at 6 and 24 h but remained constant in CRPtg mice. Thus eNOS mRNA levels were lower in CRPtg than in congenic wild-type mice 6 and 24 h after vascular injury (Fig. 2B; $P < 0.05$).

NO release. To investigate whether CRP indeed modulates NO production, we measured the levels of nitrate and nitrite in lung and heart tissues before and 6 and 24 h after vascular injury. Because NO generated by NOS has a relatively short half-life and is rapidly converted to nitrate and nitrite, measurement of nitrate/nitrite is a good indicator of NOS activity. Lung nitrate/nitrite concentration in wild-type mice was 3.0 ± 0.6 μ mol/per mg protein before injury and 3.2 ± 0.57 and 2.6 ± 0.48 μ mol/per mg protein 6 and 24 h after injury,

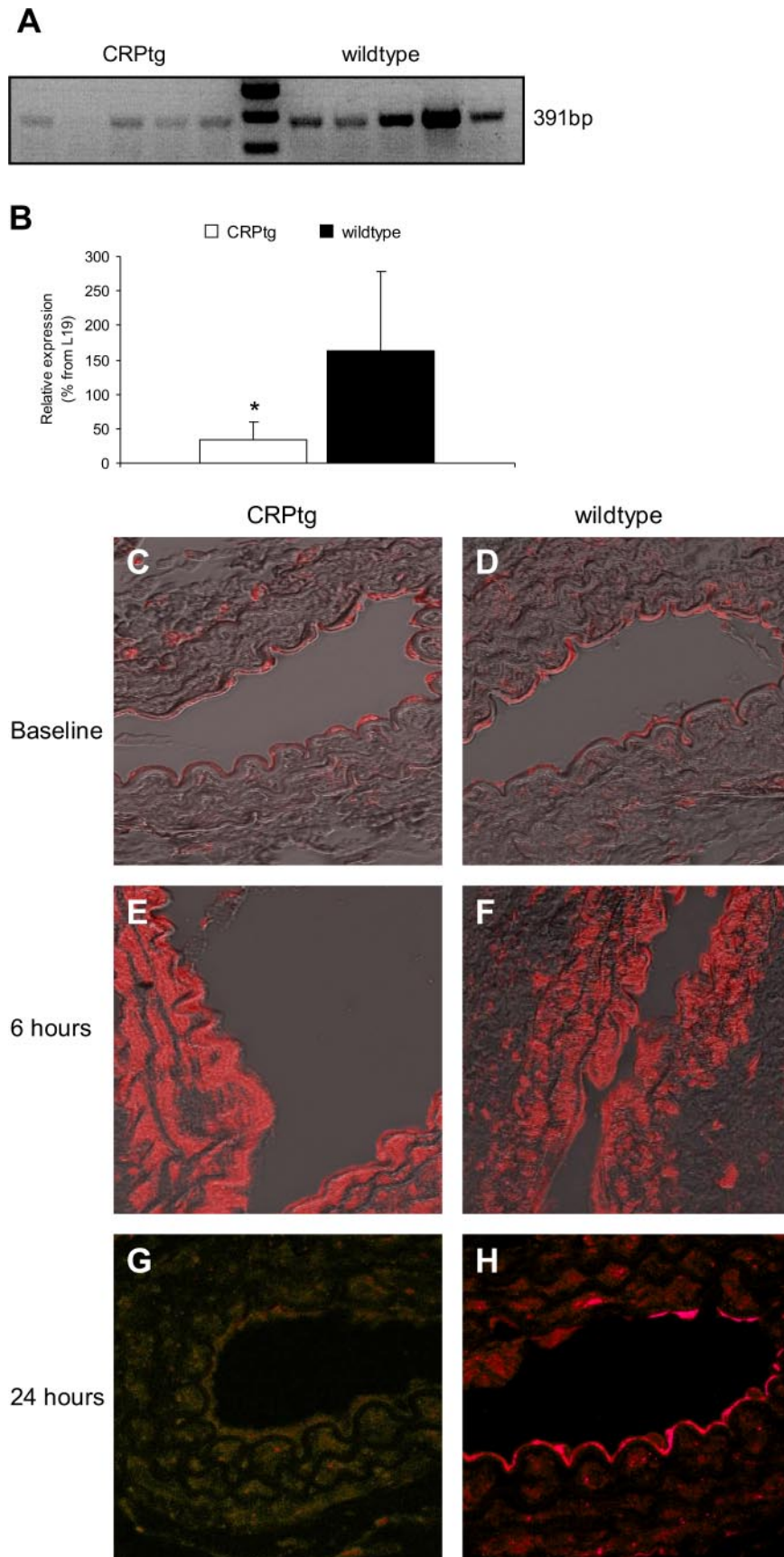
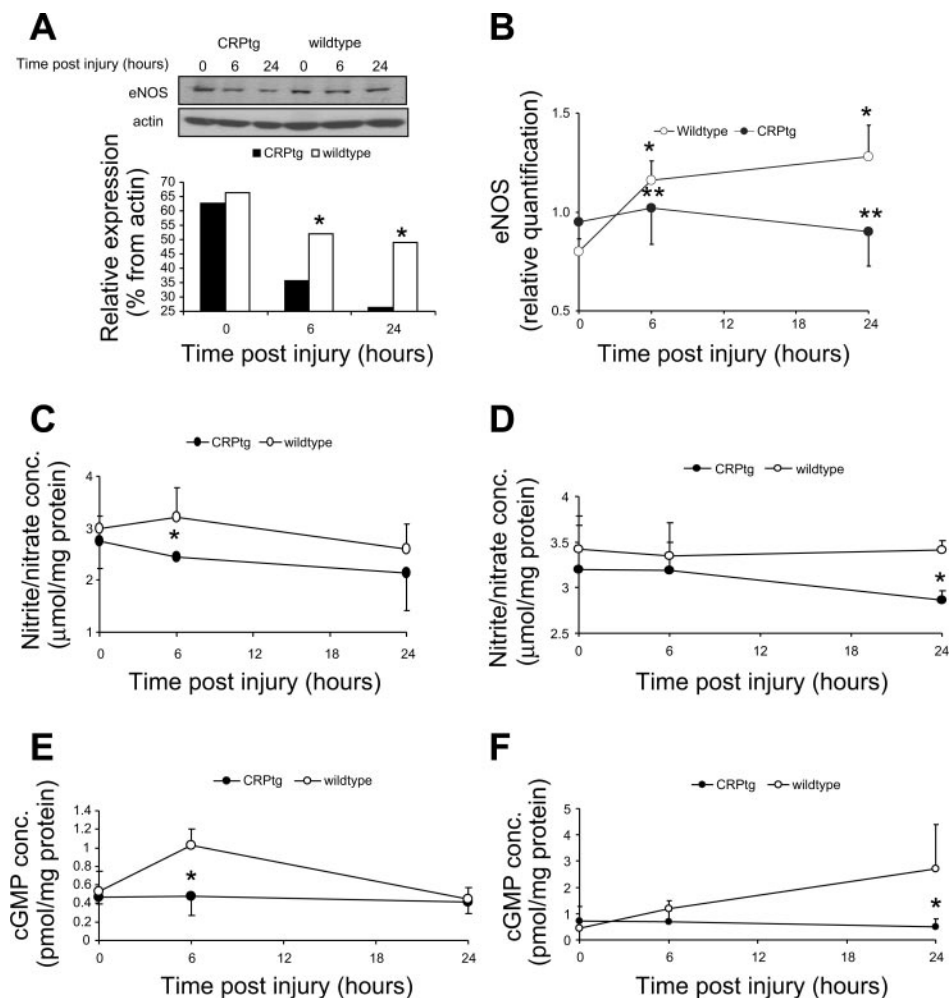


Fig. 1. C-reactive protein (CRP) suppress nitric oxide synthase (NOS) expression in injured arteries. NOS expression is lower in injured arteries of transgenic mice that express human CRP (CRPtg) than wild-type mice. *A*: semi-quantitative RT-PCR of endothelial NOS (eNOS) 24 h after injury. *B*: expression of eNOS mRNA relative to the corresponding L19 mRNA. Values are means \pm SD ($n = 5$ per group). * $P < 0.05$ vs. wild-type. *C-H*: representative images of femoral arteries from CRPtg and wild-type mice stained for eNOS 0 h (baseline), 6 h, and 24 h after injury.

Fig. 2. CRP modulates eNOS expression and activity in lung tissue. *A*: Western blot of eNOS and actin in lung of wild-type and CRPtg mice (*top*) and quantitative analysis of results of Western blot (*bottom*). CRP decreased eNOS expression 6 h after wire injury. Each lane represents protein pool from 5 animals. * $P < 0.05$ vs. CRPtg. *B*: real-time PCR for eNOS in lung before and 6 and 24 h after femoral injury in CRPtg and wild-type mice. Elevation in eNOS response to injury was suppressed in CRPtg mice. Values are means \pm SD ($n = 5$). * $P < 0.05$ vs. 0 h. ** $P < 0.05$, CRPtg vs. wild-type. *C* and *D*: CRP attenuation of NO production in lung and heart, respectively, after distant vascular injury. Nitrate and nitrite breakdown products were reduced in CRPtg after injury. Values are means \pm SD ($n = 5$). * $P < 0.01$, CRPtg vs. wild-type. *E* and *F*: CRP-induced reduction of cGMP signaling in lung and heart, respectively, after distant femoral wire injury. Values are means \pm SD ($n = 5$). * $P < 0.05$, CRPtg vs. wild-type.



respectively. Lower nitrate/nitrite concentrations in CRPtg than in wild-type mice at 0, 6, and 24 h (2.76 ± 0.28 , 2.45 ± 0.13 , and 2.14 ± 0.72 $\mu\text{mol/mg}$ protein, respectively, $n = 5$, $P < 0.05$ for 6 h; Fig. 2C) reflect lower NOS activity after injury in CRPtg mice. A similar blunted nitrite/nitrate elevation after injury was found in heart tissue (Fig. 2D; $n = 5/\text{group}$, $P < 0.05$ for 24 h).

NO signaling. NO activates soluble guanylyl cyclase, a critical component of its signaling pathway, which converts guanosine triphosphate to cGMP. To further assess the effect of CRP on NO expression, we measured cGMP levels in lung and heart tissues at baseline and 6 and 24 h after vascular injury. In accord with the blunted response for eNOS and NO breakdown products, a significant difference in cGMP was observed in the lung 6 h after distant vascular injury, with levels returning to baseline 24 h after injury ($n = 4\text{--}5/\text{group}$, $P < 0.05$ for 6 h; Fig. 2E). Blunted cGMP elevation after injury in heart tissue was similar to that in lung tissue, with a significant difference 24 h after injury (Fig. 2F).

Correlation between CRP levels and NOS expression. Plots of CRP serum levels vs. eNOS levels of expression after injury showed an inverse correlation between CRP levels and both arterial mRNA eNOS 24 h after injury ($n = 5$; Fig. 3A) and lung eNOS 6 and 24 h after injury ($n = 5$; Fig. 3, B and C).

DISCUSSION

CRP is a cardiovascular disease risk marker: high CRP levels correlate with increased occurrence of acute cardiovascular events and are probably of a higher predictive value than other known risk markers (20–22). Numerous reports support a causal link between CRP and cardiovascular pathology (for review see Ref. 36). Yet, inasmuch as most of the experiments were in tissue culture with cause-effect exhibited after the use of commercial CRP preparations, concern has been raised about spurious effects of contaminants. When impurities, in particular the preservative sodium azide and the contaminant endotoxin, are removed from commercial CRP preparations, direct endothelial activity is eliminated as well (12, 13, 29, 32, 34, 35). Transgenic animals that express human CRP or mice that express rabbit CRP (18) can be used to resolve the issue of spurious artifactual results. CRP expression in these animals is free of confounding contamination. Thus the present study examined in vivo whether CRP affects eNOS expression and activity and, indeed, supported the earlier studies by Verma et al. (34) and Venugopal et al. (32) in which recombinant human CRP was used. Expression of human CRP in transgenic mice reduces eNOS mRNA and protein expression, enzymatic bioactivity, and expected cGMP activation in injured arteries and distant organs, reflecting a local and a systemic effect.

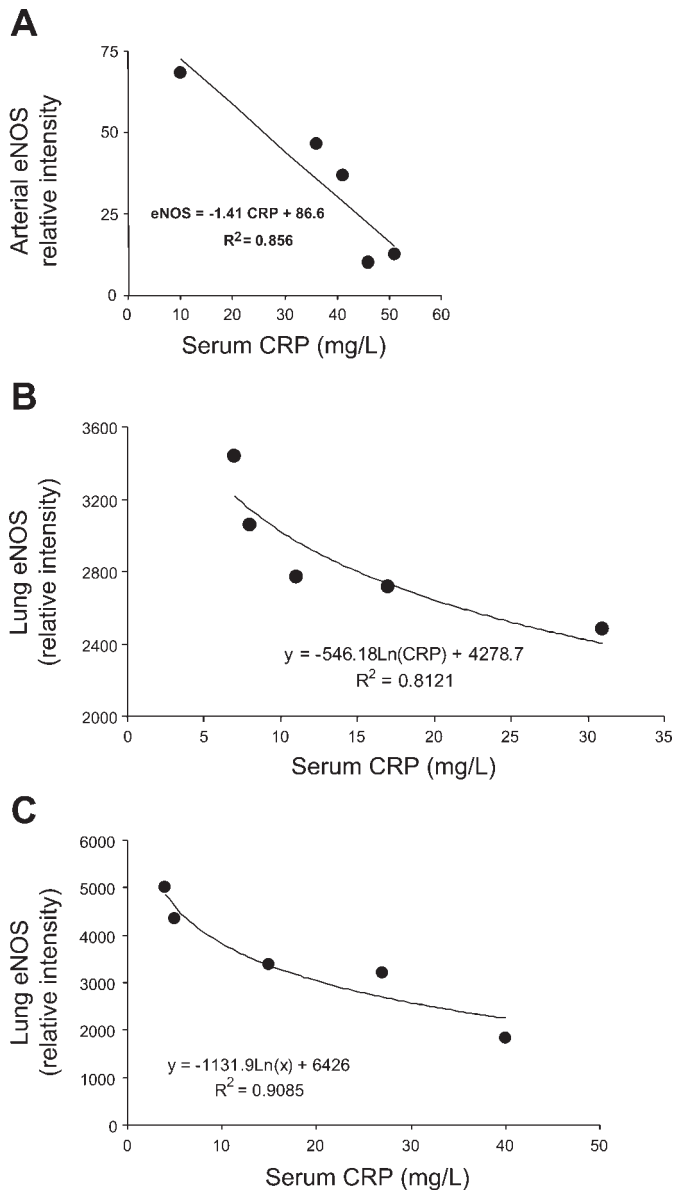


Fig. 3. CRP levels correlate with arterial and systemic NOS expression. *A*: correlation between serum CRP levels and semiquantitative RT-PCR for eNOS 24 h after wire injury ($P < 0.05$). *B* and *C*: correlation between serum CRP levels and eNOS lung protein content determined by Western blot 6 h (*B*) and 24 h (*C*) after injury ($P < 0.05$ for both). Note inverse relationship between CRP levels and local and systemic eNOS expression after vascular injury.

Furthermore, CRP levels appear to inversely correlate with eNOS expression after injury locally and systemically. These findings are further supported by the recent report of Dasu et al. (5) in which CRP inhibited eNOS activity in Toll-like (LPS) receptor knock-down endothelial cells, thus ruling out endotoxin contamination.

NO is the key endothelium-derived relaxing factor that is pivotal to the maintenance of vascular tone and reactivity. NO suppresses vasoconstriction, leukocyte adherence, platelet activation, mitogenesis, oxidation, thrombosis, and coagulation, which are associated with the pathogenesis of atherothrombotic vascular disease. Decreased NO production and endothelial dysfunction are implicated in the pathogenesis and progression

of cardiovascular disease and acute cardiovascular events (26). The suppressed NO response to injury that is observed in CRPtg mice counterbalances a major thromboregulatory mechanism. This neutralization may be the underlying cause of the accelerated thrombosis that is observed in these mice (4), suggesting a possible link between high CRP levels and the associated increased cardiovascular risk.

Arterial eNOS expression increases after arterial denudation (17). This response could not be seen at the very early stages after injury (6 h) because of the scant amount of endothelium that was left in the denuded area. The analysis of eNOS expression and activity in the distant lung and heart illustrates the fast systemic eNOS response to injury. A significant increase in eNOS expression in those organs 6 h after injury is translated into an increase in NO breakdown products, as well as an increase in cGMP signaling. By 24 h NO and cGMP levels in the lung are almost restored to baseline levels. The immediate eNOS time response is probably shared by the injured artery and expresses a fast thromboregulatory pathway. The systemic analysis of eNOS expression and activity bypasses the inherent difficulties in recording the short-term local eNOS response to arterial injury, allowing detection of the fast modulation of this response, such as that imposed by CRP.

NOS expression and NO production after injury are widespread and are not limited to the injured vessel. Changes in vascular NOS bioactivity in response to femoral artery denudation and the surgery with which it is associated were observed in the distant lung and heart. These findings are in accord with current literature, which supports a link between systemic inflammation and systemic endothelial function, i.e., NOS expression and NO production. Vascular injury exerts a systemic inflammatory response, as evidenced in our study by an abrupt increase in CRP levels. Systemic inflammatory response with an increase in CRP levels and additional modulators is inversely correlated with endothelial function as measured by vascular relaxation (8, 37). Thus the systemic vascular response is induced by local injury and aggravates further systemic response. The aggravated response after "local" injury may explain the multiplicity of vulnerable plaques in patients with acute myocardial infarctions (9). The intricate relationship between systemic inflammation and local and systemic vascular injury and repair is further complicated by recent data showing local release of CRP from vulnerable plaques and injured arteries (11). Thus systemic CRP levels may promote endothelial and vascular vulnerability, which results in further elevation of local CRP levels, which in turn further troubles the artery.

The mechanism responsible for CRP vascular activity has not been defined. The major receptor for CRP in endothelial cells is probably the Fc γ receptor. Binding and internalization of CRP by Fc γ receptors in human aortic endothelial cells appear to mediate several biological effects, including the increase in IL-8 and adhesion molecules (7). Fc γ receptor IIB was recently suggested by Mineo et al. (14) to modulate CRP-induced eNOS suppression and the subsequent endothelial dysfunction. Mineo et al. reported that CRP nongenomically blunted eNOS phosphorylation in cultured endothelium. We have shown that in vivo CRP suppresses eNOS mRNA expression, suggesting an additional genomic pathway for CRP suppression of eNOS. Further studies are warranted to examine whether modulation of Fc γ receptor IIB in vivo will affect

eNOS transcription as well as the prothrombotic phenotype of CRP transgenic mice.

Mice are considered useless for the study of CRP functions, since CRP is not an acute-phase reactant in mice. The human CRP transgenic mouse was generated to overcome this problem. Human CRP is a foreign antigen in the mouse, and there are many uncertainties concerning its functional role in the immune system of these animals. The situation is complicated further when these mice are used for studies of atherogenesis because of the lack of human-like interaction between CRP, complement, and LDL in these mice (18, 30, 36). The present study demonstrates significant changes in eNOS expression in CRP_{Tg} compared with congenic wild-type mice. These changes are in accord with *in vitro* studies as well as *in vivo* data showing CRP-induced blunted NO-mediated acetylcholine-induced arterial conductance (14). However, additional studies are warranted to further elucidate the mechanisms by which CRP modulates eNOS expression and the relevance to humans.

In conclusion, the complex relationship between the inflammatory response and vascular injury and repair is of major importance in the pathogenesis of cardiovascular disease. CRP is not only a strong marker for cardiovascular morbidity but, also, a modulator that suppresses NOS expression and bioactivity in response to vascular injury. The vascular effect of CRP extends beyond the injured artery, with NOS suppression observed in distant vascular tissues. Thus CRP not only hampers the local compensatory and reparative processes of injury but, also, may affect and destabilize distant vessels as well.

GRANTS

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