short communication

The biological effects of CRP are not attributable to endotoxin contamination: evidence from TLR4 knockdown human aortic endothelial cells

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Abstract C-reactive protein (CRP) is the prototypic marker of inflammation and a strong predictor of cardiovascular events in humans. There are questions regarding the validity of the biological effects reported for CRP, in spite of adherence to rigorous control measures minimizing endotoxin [lipopolysaccharide (LPS)] contamination in these in vitro studies. In this study, we addressed the key question of endotoxin contamination in CRP preparations using Toll-like receptor 4 (TLR4) knockdown endothelial cells. Human aortic endothelial cells (HAECs) transfected with prevalidated TLR4 small interfering RNA (siRNA) and scrambled siRNA controls were challenged with pleural fluid-derived CRP or LPS for 12–16 h. Secreted interleukin-6 (IL-6), IL-1β, IL-8, and plasminogen activator inhibitor-1 (PAI-1) levels and endothelial Nitric oxide synthase (eNOS) activity were determined. TLR4 knockdown in HAECs significantly decreased LPS-induced IL-1β, IL-6, and IL-8, whereas the stimulatory effects of CRP were similar in both scrambled control and TLR4 knockdown cells. Furthermore, CRP significantly stimulated PAI-1 levels in both control and TLR4-transfected cells and inhibited eNOS activity, whereas LPS effects were negated in TLR4-transfected cells. The data presented cogently demonstrate and further confirm that the biological effects of CRP on HAECs are independent of LPS and thus are attributable to native protein per se. This is the first study to positively authenticate the significance of earlier in vitro reports on CRP biological effects.—Dasu, M. R., S. Devaraj, T. W. Du Clos, and I. Jialal. **The biological effects of CRP are not attributable to endotoxin contamination: evidence from TLR4 knockdown human aortic endothelial cells. *J. Lipid Res.* 2007.** 48: **509–512.**

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C-reactive protein (CRP) is the prototypic marker of inflammation in human. Studies in healthy volunteers established CRP as a strong predictor of cardiovascular events. In addition, CRP appears to mediate atherothrombosis (1–4). CRP has been shown to induce cell adhesion molecules, chemokines, PAI-1, and endothelin-1 and to inhibit the expression and bioactivity of eNOS, prostacyclin, and tissue Plasminogen activator (tPA) in endothelial cells (5). Furthermore, CRP also induces reactive oxygen species, cytokines, and tissue factor in monocytes (6). As reviewed recently, although the majority of the proinflammatory/prothrombotic effects known have been reported in endothelial cells, CRP has effects on monocytes-macrophages and smooth muscle cells as well (5). There are questions regarding the validity of the CRP biological effects reported in spite of adherence to rigorous quality-control measures minimizing endotoxin [lipopolysaccharide (LPS)] contamination in these in vitro studies. Moreover, the reliability of in vitro studies using commercial CRP preparations was questioned by several investigators because of these confounding variables (7–9).

In this study, we addressed the key question of endotoxin contamination. We tested the effects of purified CRP on Toll-like receptor 4 (TLR4) small interfering RNA (siRNA)-transfected human aortic endothelial cells (HAECs), because the majority of biological effects have been reported in endothelial cells. TLR4 is a transmembrane LPS-sensing molecule with a cytoplasmic domain for proinflammatory signaling, and recognition by the receptors is linked directly to inflammatory responses (10) and is pathogenically related to atherosclerosis (11). We provide evidence that TLR4 knockdown has no influence on CRP biological effects via interleukin-8 (IL-8), IL-6, IL-1β, PAI-1, and cGMP levels as well as eNOS activity, convincingly demonstrating that these effects are attributable to the native pentameric protein and not to endotoxin contamination.

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MATERIALS AND METHODS

Cell culture and human CRP purification

HAECs (Clonetics, San Diego, CA) were cultured as described previously (12) and were used within five passages for all experiments. CRP was purified from human pleural or ascites fluid by the method of Du Clos, Zlock, and Marnell (13). LPS was removed from CRP preparations with a Detoxigel column (Pierce Biochemicals) and found to contain <0.125 EU/ml (<12.5 pg/ml) by the Limulus assay (Biowhittaker), as described previously (12). Medium with endotoxin levels of <0.125 EU/ml was used in all experiments. CRP protein was concentrated using Amicon concentrators (Amicon, Beverly, MA), dialyzed against Tris-buffered saline with 2 mM calcium, sterile-filtered, and stored at 4°C. None of the buffers contained sodium azide. The purity of pentameric CRP was checked by native gel electrophoresis under nonreducing conditions, yielding a single band at ~120 kDa, as described previously (14), and was consistently used in all experiments.

siRNA transfection

Cells (1×10^5 cells/ml/well; 12-well plate) were transiently transfected with prevalidated TLR4 siRNA (10–20 pmol/well; GenBank accession number NM_138554) using siPORT amine reagent according to the manufacturer's instructions (Ambion, Austin, TX) in serum-free medium with suitable scrambled siRNA control. Forty-eight hours later, transfected cells were challenged with CRP (25 μg/ml) or LPS (100 pg/ml, 1 ng/ml, or 1 μg/ml) for 12–16 h. LPS (*Escherichia coli* 026:B6; Sigma Chemicals, St. Louis, MO) was used as a positive control. Additionally, a set of plates with siPORT amine (vehicle control) and untransfected (control) treated similarly were run in parallel with all experiments (these data were similar to scrambled control data and are not shown).

RT-PCR, ELISA, and eNOS bioactivity

Cell supernatants, lysates, and RNA were collected for ELISA, eNOS activity, and RT-PCR, respectively, at the end of the treatment. Cell viability, assessed by the 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium Bromide assay, was >95% with this dose range of CRP and LPS. TLR4 knockdown was measured using sequence-specific primers from Invivogen by RT-PCR. TLR4 knockdown efficiency was consistently between 65% and 75%. Secreted IL-6, IL-1β, and IL-8 protein, PAI-1 antigen, and eNOS enzyme activity were measured by assaying [^{3}H]L-arginine conversion to [^{3}H]L-citrulline in HAEC lysates as described previously (15, 16). Also, as a measure of eNOS bioactivity, total cGMP levels were measured as described previously (12). The interassay and intra-assay coefficient of variation for these assays was <10%. Data are expressed as means ± SD. Significance was defined as $P < 0.05$ using Student's *t*-test.

RESULTS

TLR4 knockdown in HAECs was assessed using mRNA levels by RT-PCR as the readout (**Fig. 1A**). Significant reduction of the TLR4/GAPDH mRNA ratio was detected compared with the scrambled control ($P < 0.05$) (Fig. 1B). As shown in **Table 1**, TLR4 knockdown in HAECs significantly decreased LPS-induced IL-1β, IL-6, and IL-8 secretion ($P < 0.05$), whereas stimulatory effects of CRP were similar in both scrambled control and TLR4 knockdown cells. Furthermore, CRP significantly stimulated PAI-1 levels in both control and TLR4 siRNA-transfected cells, whereas LPS effects were negated in TLR4 siRNA-transfected cells. Also, although TLR4 knockdown had no effect on CRP-inhibited eNOS activity as assessed by [^{3}H]L-citrulline release in cell lysates (scrambled, 110 ± 3 pmol/mg protein/min; scrambled + CRP, 40 ± 1; TLR4, 102 ± 4; TLR4 + CRP, 40 ± 0.5) and bioactivity of eNOS assessed by cGMP levels (scrambled, 446 ± 2 fmol/mg protein; scrambled + CRP, 144 ± 3; TLR4, 448 ± 11; TLR4 + CRP, 179 ± 3), LPS failed to show any effect on eNOS and cGMP in scrambled siRNA-transfected cells (data not shown). Lower doses of LPS (100 pg/ml and 1 ng/ml) showed similar biological effects as 1 μg/ml LPS on TLR4 knockdown cells in all assays (data not shown).

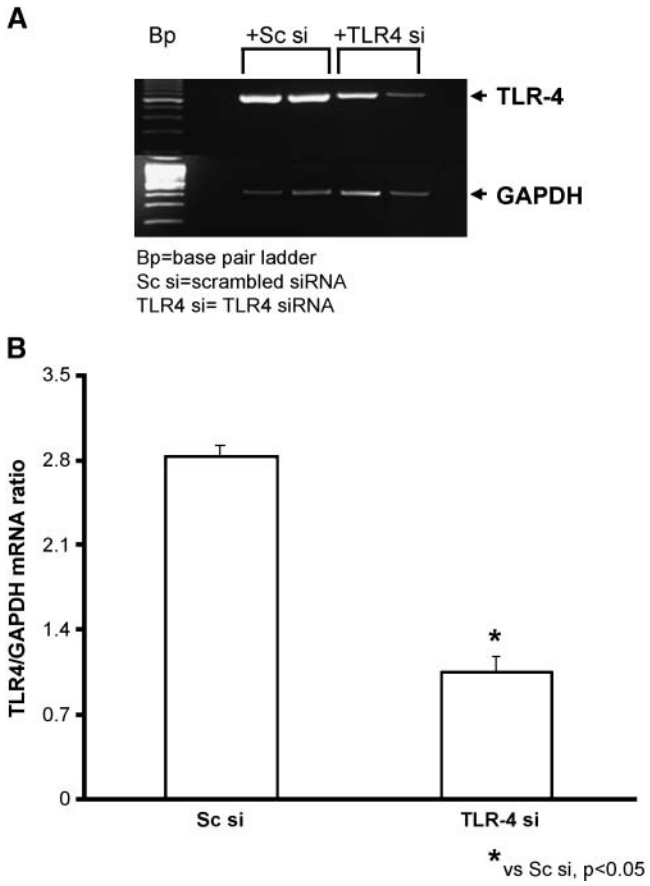

Fig. 1. A: Toll-like receptor 4 (TLR4) small interfering RNA (siRNA) knockdown efficiency was measured using RT-PCR. Human aortic endothelial cells were transfected with TLR4 and scrambled siRNA (Sc si). One microgram of total RNA was used to make cDNA. PCR was performed with 50–100 ng of cDNA to detect TLR4 and GAPDH mRNA using sequence-specific primers (Invitrogen). TLR4 mRNA levels were suppressed compared with those of the scrambled control. GAPDH mRNA levels were unaffected by the transfection. Each panel is representative of four separate experiments. B: Densitometric analysis of TLR4 mRNA knockdown in endothelial cells. The ratio of TLR4/GAPDH mRNA was decreased significantly compared with that of the scrambled control. Each bar represents the average ± SD of two band intensities (n = 4 separate experiments; * $P < 0.05$).

TABLE 1. Biological effects of CRP and LPS on TLR4 and scrambled siRNA-transfected human aortic endothelial cells

Sample	Treatment	+Scrambled siRNA	+TLR4 siRNA
IL-1β	Control	130 ± 10	101 ± 17
	CRP, 25 μg/ml	373 ± 17[a]	295 ± 18
	LPS, 1 μg/ml	342 ± 19[a]	26 ± 8[b]
IL-6	Control	1,383 ± 531	879 ± 66
	CRP, 25 μg/ml	4,345 ± 1,634[a]	6,032 ± 474
	LPS, 1 μg/ml	34,144 ± 12,872[a]	620 ± 78[b]
IL-8	Control	142 ± 36	160 ± 60
	CRP, 25 μg/ml	653 ± 36[a]	535 ± 32
	LPS, 1 μg/ml	722 ± 34[a]	98 ± 21[b]
PAI-1	Control	16 ± 1.6	7.4 ± 1.8
	CRP, 25 μg/ml	28 ± 1.1[a]	25 ± 1.4
	LPS, 1 μg/ml	29 ± 2.6[a]	5.9 ± 1.1[b]

CRP, C-reactive protein; IL, interleukin; LPS, lipopolysaccharide; siRNA, small interfering RNA; TLR4, Toll-like receptor 4. Data are reported as secreted levels (pg/mg protein; means ± SD).

[a] $P < 0.05$ versus control (paired *t*-test); n = 4 separate experiments in duplicate.

[b] $P < 0.05$ versus scrambled (paired *t*-test); n = 4 separate experiments in duplicate.

DISCUSSION

In vitro studies investigating the proatherogenic role of CRP have been criticized for the use of commercial preparations of CRP, which may be partially defined and may contain endotoxin. In this context, we investigated the biological properties of our in-house-purified CRP preparations (LPS levels < 0.125 EU/ml; derived from pleural/ascites fluid and devoid of sodium azide) in TLR4 siRNA-transfected endothelial cells. TLR4 was the first member of the TLR family to be characterized and identified as critical for LPS recognition (17). Although some reports indicate TLR2 as an endotoxin sensor (18), Beutler (19) and others have convincingly established that TLR2 makes no contribution to endotoxin signal transduction. Therefore, the use of TLR4 siRNA to rule out the role of endotoxin in this study was appropriate. Although demonstration of CRP's biological effects in vivo in TLR4/Myd88 knockout mice would be ideal, human CRP does not appear to exert proinflammatory/prothrombotic effects in mice (5). Hence, the use of TLR4 knockdown HAECs is optimal to test the effects of CRP per se.

Several facts exclude the possibility that LPS contamination is responsible for the reported biological effects of CRP in our study. First, we noted no inflammatory effects of LPS in TLR4-suppressed endothelial cells, which is the receptor for sensing LPS and consequent signaling. Second, the amount of LPS used as a positive control (1 μg/ml) was sufficient to induce an inflammatory response in endothelial cells, which was consistently absent in TLR4 knockout cells. Last, the amount of LPS in our human CRP preparation was 0.125 EU/ml, whereas the critical amount to induce the activation of coagulation and/or cytokine release exceeds 20 EU/ml (20). In this report, even at the highest concentration of LPS (1 μg/ml), TLR4 knockdown in endothelial cells failed to demonstrate any of its biological effects.

These data demonstrate conclusively that the biological responses to CRP stimulation are not attributable to contaminating LPS. This argues that the responses to CRP are most likely attributable to the pentameric CRP, which makes up all of the recognizable elements in the preparation. Previous studies on the effects of CRP on monocytes-macrophages and endothelial cells ruled out any role of LPS in its biological functions (21). Recently, using a selection of strategies, we examined CRP biological effects via a broad range of control experiments (trypsin digestion of CRP, boiling CRP, preabsorbing CRP on plates coated with anti-CRP IgG, polymyxin B pretreatment, etc.) and determined the specificity of human CRP (16). Also, using robust specificity controls both in vitro and in vivo, we reported that CRP downregulates LPS-stimulated IL-10 secretion in human monocyte-derived macrophages via cAMP and phospho cAMP Response Element Binding protein, indicating that CRP's effects are independent of LPS (22). In addition, we previously established that CRP mediates its biological effects on HAECs through Fcγ receptors, CD32 and CD64 (23), using specific antibodies and siRNAs. Bisoendial, Kastelein, and Stroes (24) provided further in vivo evidence supporting the proinflammatory effects of CRP in humans. Our data confirm that the biological effects of CRP on HAECs are independent of endotoxin, which is a common contaminant in commercial CRP preparations. In conclusion, this is the first study to positively authenticate the significance of earlier in vitro reports on the biological effects of CRP.

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REFERENCES

1. Libby, P., P. M. Ridker, and A. Maseri. 2002. Inflammation and atherosclerosis. *Circulation.* **105:** 1135–1143.
2. Ridker, P. M. 2001. High-sensitivity CRP: potential adjunct for global risk assessment in the primary prevention of cardiovascular disease. *Circulation.* **103:** 1813–1818.
3. Jialal, I., and S. Devaraj. 2001. Inflammation and atherosclerosis: the value of the high-sensitivity C-reactive protein assay as a risk marker. *Am. J. Clin. Pathol.* **116 (Suppl.):** 108–115.
4. Yeh, E. T., and J. T. Willerson. 2003. Coming of age of C-reactive protein: using inflammation markers in cardiology. *Circulation.* **107:** 370–371.
5. Verma, S., S. Devaraj, and I. Jialal. 2006. Is C-reactive protein an innocent bystander or proatherogenic culprit? C-reactive protein promotes atherothrombosis. *Circulation.* **113:** 2135–2150.
6. Cermak, J., N. S. Key, R. R. Bach, J. Balla, H. S. Jacob, and G. M. Vercellotti. 1993. CRP induces human peripheral blood monocytes to synthesize tissue factor. *Blood.* **82:** 513–520.
7. Pepys, M. B., P. N. Hawkins, M. C. Kahan, G. A. Tennent, J. R. Gallimore, D. Graham, C. A. Sabin, A. Zychlinsky, and J. de Diego. 2005. Proinflammatory effects of bacterial recombinant human C-reactive protein are caused by contamination with bacterial products, not by C-reactive protein itself. *Circ. Res.* **97:** e97–e103.
8. Pepys, M. B. 2005. CRP or not CRP? That is the question. *Arterioscler. Thromb. Vasc. Biol.* **25:** 1091–1094.
9. Liu, C., S. Wang, A. Deb, K. A. Nath, Z. S. Katusic, J. P. McConnell, and N. M. Caplice. 2005. Proapoptotic, antimigratory, antiproliferative, and antiangiogenic effects of commercial C-reactive protein on various human endothelial cell types in vitro: implications of contaminating presence of sodium azide in commercial preparations. *Circ. Res.* **97:** 135–143.
10. Beutler, B. 2002. TLR4 as the mammalian endotoxin sensor. *Curr. Top. Microbiol. Immunol.* **270:** 109–120.

11. Mullick, A. E., P. S. Tobias, and L. K. Curtiss. 2006. Toll-like receptors and atherosclerosis: key contributors in disease and health? *Immunol. Res.* **34:** 193–209.
12. Venugopal, S. K., S. Devaraj, I. Yuhanna, P. Shaul, and I. Jialal. 2002. Demonstration that C-reactive protein decreases eNOS expression and bioactivity in human aortic endothelial cells. *Circulation.* **106:** 1439–1441.
13. Du Clos, T. W., L. T. Zlock, and L. Marnell. 1991. Definition of a C-reactive protein binding determinant on histones. *J. Biol. Chem.* **266:** 2167–2171.
14. Devaraj, S., S. Venugopal, and I. Jialal. 2006. Native pentameric C-reactive protein displays more potent pro-atherogenic activities in human aortic endothelial cells than modified C-reactive protein. *Atherosclerosis.* **184:** 48–52.
15. Devaraj, S., D. Y. Xu, and I. Jialal. 2003. C-reactive protein increases plasminogen activator inhibitor-1 expression and activity in human aortic endothelial cells: implications for the metabolic syndrome and atherothrombosis. *Circulation.* **107:** 398–404.
16. Singh, U., S. Devaraj, and I. Jialal. 2005. C-reactive protein decreases tissue plasminogen activator activity in human aortic endothelial cells: evidence that C-reactive protein is a procoagulant. *Arterioscler. Thromb. Vasc. Biol.* **25:** 2216–2221.
17. Hoshino, K., O. Takeuchi, T. Kawai, H. Sanjo, T. Ogawa, Y. Takeda, K. Takeda, and S. Akira. 1999. Cutting edge. Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the LPS gene product. *J. Immunol.* **162:** 3749–3752.
18. Kirschning, C. J., H. Wesche, T. M. Ayres, and M. Rothe. 1998. Human Toll-like receptor 2 confers responsiveness to bacterial lipopolysaccharide. *J. Exp. Med.* **188:** 2091–2097.
19. Beutler, B. 2000. Tlr4: central component of the sole mammalian LPS sensor. *Curr. Opin. Immunol.* **12:** 20–26.
20. Levi, M., H. ten Cate, K. A. Bauer, T. van der Poll, T. S. Edgington, H. R. Buller, S. J. Van Deventer, C. E. Hack, J. W. ten Cate, and R. D. Rosenberg. 1994. Inhibition of endotoxin-induced activation of coagulation and fibrinolysis by pentoxifylline or by a monoclonal anti-tissue factor antibody in chimpanzees. *J. Clin. Invest.* **93:** 114–120.
21. Jialal, I., S. Devaraj, and S. K. Venugopal. 2004. C-reactive protein: risk marker or mediator in atherothrombosis? *Hypertension.* **44:** 6–11.
22. Singh, U., S. Devaraj, M. R. Dasu, D. Ciobanu, J. Reusch, and I. Jialal. 2006. C-reactive protein decreases interleukin-10 secretion in activated human monocyte-derived macrophages via inhibition of cyclic AMP production. *Arterioscler. Thromb. Vasc. Biol.* **26:** 2469–2475.
23. Devaraj, S., T. W. Du Clos, and I. Jialal. 2005. Binding and internalization of C-reactive protein by Fcgamma receptors on human aortic endothelial cells mediates biological effects. *Arterioscler. Thromb. Vasc. Biol.* **25:** 1359–1363.
24. Bisoendial, R., J. Kastelein, and E. Stroes. 2005. In response to van den Berg et al: on the direct actions of CRP in humans. *Circ. Res.* **97:** e71.