BIOPHYSICS AND BIOCHEMISTRY

Effect of C-Reactive Protein on the Production of Nitric Oxide by Mouse Peritoneal Macrophages

Yu. V. Shebzukhov, M. Yu. Vaisburd, K. V. Artyushkin, and E. B. Mysyakin

Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 125, No. 1, pp. 48-50, January, 1998
Original article submitted December 20, 1996

C-reactive protein in concentrations of 1-20 mg/ml induces the production of nitrogen oxide by mouse peritoneal macrophages attracted by peptone solution. Protein kinases A and C and RNA and protein production inhibitors abolish this effect, while inhibitors of the lipoxigenase and cycloxigenase pathways of arachidonic acid metabolism attenuate it.

Key Words: *C-reactive protein; nitrogen oxide; macrophages; intracellular signaling*

The mechanisms by which C-reactive protein (CRP) participates in defense of the organism are still unknown [13]. As the main reagent of the acute phase response, this protein with a molecular weight of about 115 kD is produced by human and animal hepatocytes. It activates the classical pathway of complement activation and opsonizes bacteria, injured cells and tissues, and their fragments [5,7,13].

Negligible amounts of CRP are produced by Kupffer's cells and peripheral blood leukocytes [4,8-10]. CRP produced by these cells is exposed on the external surface of plasma membrane and is retained there due to reactions with specific CRP-binding protein. This membrane-bound form of CRP can serve as a receptor of galactose residues and play the role of a selectin-like adhesion molecule [4,8,9].

Specific CRP-binding proteins were detected on Kupffer's cells, monocytes, macrophages, and in many myelomonocyte and macrophage-like cell strains [2,4,16]. CRP-binding sites can be represented by one or two proteins with different affinity for the ligand, depending on the source of cells. Proteins with molecular weight of 57-61 kD are among CRP-binding molecules detected on all above-mentioned mononuclear phagocytes. The number of CRP-binding sites varies from 5.5×10^4 to 3.6×10^5per cell, and K_d from 1.25×10^{-8} to 1.1×10^{-7} M [2,4,15,16].

CRP regulates many functions of mononuclear phagocytes: it modulates respiratory burst caused by some antigens, initiates the production of colony-stimulating and tissue factors, and stimulates phagocytosis and antitumor properties of monocytes and macrophages [3,12-14].

We investigated the effect of CRP on macrophagal production of nitric oxide (NO), a low-molecular-weight compound which exhibits bactericidal and cytotoxic activities and participates in intracellular signaling [6].

MATERIALS AND METHODS

Human CRP was obtained as described previously [1]. Peritoneal macrophages of (CBA×C57BL/6) F_1 mice attracted 3 days after intraperitoneal injection of 2 ml of 4% peptone solution (Difco) were washed from the abdominal cavity with normal saline and enriched by adhesion to the plastic for 1 h at 37°C in an atmosphere with 5% CO_2 in RPMI-1640 with 1 mM HEPES, 2 mM L-glutamine, and 80 mg/liter gentamicin (all components from Flow) with 5% fetal calf serum.

N. F. Gamaleya Institute of Epidemiology and Microbiology, Russian Academy of Medical Sciences, Moscow

0007-4888/98/0001-0040$20.00©1998 Plenum Publishing Corporation

The cells were pipetted in 96-well plates (Flow), 2×10^5cells/well in 200 μl RPMI-1640 with the above additives and 5% fetal calf serum in the presence of the compounds listed further. The cells were pre-incubated for 30 min at 37°C in an atmosphere with 5% CO_2 with the inhibitor. After 24-h incubation at 37°C in atmosphere with 5% CO_2, NO production by macrophages was assessed by accumulation of NO_2^- in the culture medium. For this, 100 μl of culture medium was transferred into a well of a 96-well plate and 100 μl 1.5% sulfonylamide in 1 N HCl and 100 μl 0.15% N-(1-naphthyl)ethylenediamine in H_2O were added in succession, the mixture was incubated for 15-30 min at ambient temperature, and light absorbance was measured at 540-570 nm. Bacterial lipopolysaccharide (LPS) was detected, and its concentration measured by the LAL test (E-Toxate, Sigma).

RESULTS

CRP in concentrations of 1-20 μg/ml induced high production of NO by peptone-attracted mouse peritoneal macrophages *in vitro* (Fig. 1). NO production was maximal at a CRP concentration of 10 mg/ml: 39.7± 9.4 μM NO/10^6 cells/ml. Further increase in CRP concentration led to a decrease in NO production.

To elucidate the mechanisms contributing to the transfer of activation signals from CRP binding centers to NO-producing cell structures, macrophages were treated with agents inhibiting intracellular signaling (Table 1).

It was found that inducible NO-synthase (iNOS) was produced by macrophages. This enzyme converts L-arginine in citrullin and releases NO during this process. Essential stages in the production of iNOS are transcription of iNOS mRNA with subsequent enzyme production, which are abolished by actinomycin D and cycloheximide, respectively.

Abolition of this effect by staurosporin and H-9 indicates that protein kinase C participates in the transfer of activation signal from CRP receptor to NO-producing cell structures. Attenuation of the effect by lipoxigenase (NDGA, nordihydroguaretic acid) and cycloxygenase (indomethacin) indicates that arachidonic acid metabolites are involved in stimulation of NO production.

For identifying the type of protein kinases activated during CRP reaction with macrophages, we investigated the relationship between suppression of NO production and concentrations of saurosporin and H-9 (Fig. 2, *a*, *b*). ID_{50} for saurosporin was 50±18 nM and for H-9 3.2±1.1 μM. Therefore, protein kinases A and C may be activated during reaction of CRP with macrophages. It is not clear

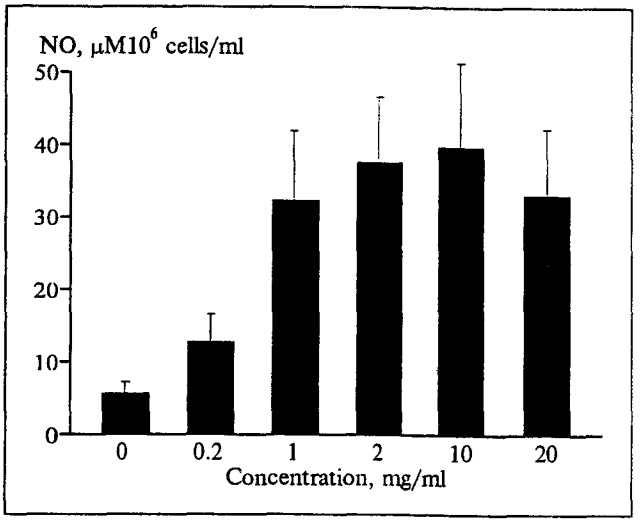

Fig. 1. Effect of C-reactive protein on NO production by peritoneal macrophages of (CBA×C57BL/6) F_1 mice.

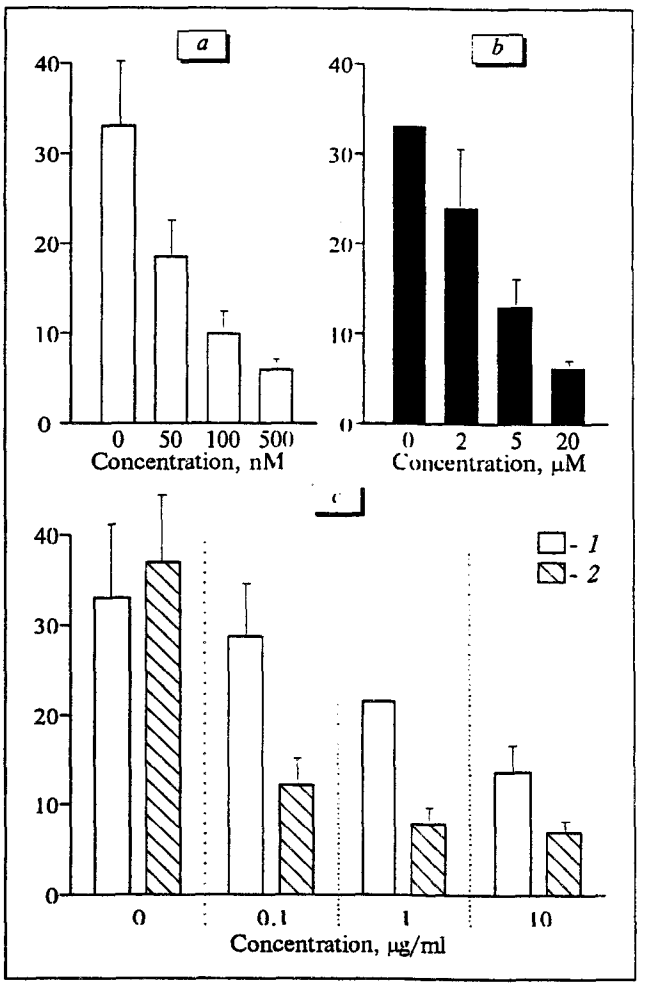

Fig. 2. Effects of staurosporin (*a*), H-9 (*b*), and polymixin B (*c*) on macrophagal NO production induced by C-reactive protein (20 μg/ml). Ordinate: NO production, μM/10^6cells/ml. *b*: *1*) C-reactive protein, 20 μg/ml; 2) lipopolysaccharide, 100 ng/ml.

TABLE 1. Effects of Inhibitors of Intracellular Signaling on CRP (20 mg/ml) Induced Production of NO by Mouse Peritoneal Macrophages (*M±m*)

Pretreatment	NO production, μM/10^6 cells/ml
Control without stimulation	5.55±1.30
Control with stimulation	33.00±7.80
Actinomycin D, 100 nM	7.30±1.80
Cycloheximide, 100 nM	8.10±1.95
Staurosporin, 500 nM	6.00±1.45
H-9, 20 μM	6.20±1.50
NDGA, 10 μM	22.40±5.40
Indomethacin, 100 nM	21.90±5.25
NDGA, 10 μM+ indomethacin, 100 nM	11.10±3.40

whether activation of protein kinase C results from reaction of CRP with specific receptors on the surface of macrophages or from some other process. Recently, CRP was shown to hydrolyze the plasma membrane lipid phosphatidylcholine and release diacylglycerol, thus creating conditions for activation of protein kinase C [11].

Since bacterial LPS is a potent stimulant of NO production by macrophages, we measured LPS admixtures in CRP preparations and assessed their contribution to CRP-induced NO production by macrophages. LAL test (E-Toxate) suggested an endotoxin admixture in CRP preparations in quantities of about 1 mg LPS per 1.6 mg CRP. Unfortunately, the presence of LAL test inhibitors in CRP preparations does not permit a more precise assessment of endotoxin admixture.

For assessing the contribution of LPS admixture, we compared the effect of polymixin B on macrophagal NO production induced by CRP (20 mg/ml) and LPS (100 ng/ml). Polymixin in a dose of 100 ng/ml decreased LPS-induced production of NO by 80% (Fig. 2, *c*) and CRP-induced production of NO (ID_{50}=1.6±0.6 mg/ml). In addition to inactivation of lipid A (active LPS component), polymixin B inhibits protein kinase C. Therefore, the decrease in CRP-induced production of NO is one more proof of protein kinase C activation during CRP interaction with macrophages.

Thus, we have supplemented the list of NO production inductors with one more compound, CRP, and shown the intracellular mechanisms contributing to the realization of this effect.

REFERENCES

1. E. G. Zotova, I. B. Druzhinina, S. Zh. Toksambaeva, *et al.*, *Biotekhnologiya*, No. 3-4, 15-19 (1995).
2. S. P. Ballou, J. Buniel, and S. S. Macintyre, *J. Immunol.*, **142**, No. 8, 2708-2713 (1989).
3. J. Cofta, J. K. Lacki, S. H. Mackiewicz, and K. E. Wiktorowicz, *Int. J. Immunopharmacol.*, **8**, No. 2, 95-102 (1995).
4. C. Egenhofer, K. Alsdorff, K. Fehsel, and V. Kolb-Bachofen, *Hepatology*, **18**, No. 5, 1216-1223 (1993).
5. H. Gewurz, X. H. Zhang, and T. F. Lint, *Curr. Opin. Immunol.*, **7**, No. 1, 54-64 (1995).
6. R. Gopalakrishna, Z. H. Chen, and U. Gundimeda, *J. Biol. Chem.*, **286**, No. 36, 27180-27185 (1993).
7. J. Hurlimann, G. J. Thorbecke, and G. M. Hochwald, *J. Exp. Med.*, **123**, 365-378 (1966).
8. G. Kempka, P. H. Roos, and V. Kolb-Bachofen, *J. Immunol.*, **144**, No. 3, 1004-1009 (1990).
9. V. Kolb-Bachofen, N. Puchtateudt, and C. Egenhofer, *Glycoconj. J.*, **12**, No. 2, 122-127 (1995).
10. A. E. Kuta and L. L. Baum, *J. Exp. Med.*, **164**, No. 7, 321-326 (1986).
11. S. Mookerjea and D. Hunt, *Biochem. Biophys. Res. Commun.*, **208**, No. 3, 1046-1052 (1995).
12. M. Ramani, F. Khechai, V. Ollivier, *et al.*, *FEBS Lett.*, **356**, No. 1, 86-88 (1994).
13. M. J. Shields, *Immunol. Res.*, **12**, No. 1, 37-47 (1993).
14. P. P. Singh, S. Singh, G. P. Dutta, and N. B. Singh, *Eur. Cytokine Netw.*, **6**, No. 1, 37-43 (1995).
15. J. M. Tebo and R. F. Mortensen, *J. Immunol.*, **144**, No. 1, 231-238 (1990).
16. K. Zahedi, J. M. Tebo, J. Siripont, *et al.*, *Ibid.*, **142**, No. 7, 2384-2392 (1989).