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C-reactive protein decreases protein phosphorylation in stimulated human neutrophils

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Treatment of human neutrophils with C-reactive protein (CRP) causes a concentration-dependent decrease in the extent of activation of superoxide production and of granule secretion, induced by phorbol-12-myristate-13-acetate (PMA) or *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLF). The same treatment also causes a significant reduction in the degree of PMA- and fMLF-stimulated phosphorylation of several cell proteins. These include the proteins of 43–47 kDa, whose extent of phosphorylation correlates with the activation of superoxide production and of secretion. Contrary to the effects exerted on protein phosphorylation, CRP does not affect the fMLF-elicited increase in neutrophil cytosolic Ca^{2+}.

C-reactive protein; Neutrophil; Protein phosphorylation; Superoxide; Secretion

1. INTRODUCTION

C-reactive protein is the prototype acute-phase serum protein, whose concentration increases from a median of 1 μg/ml in healthy subjects up to 1000-fold during inflammatory situations [1]. Although CRP can influence some functions of lymphocytes [2] and macrophages [3], neutrophils are the most likely target of CRP. Actually, deposition of CRP mainly occurs in vivo at inflammatory sites infiltrated with neutrophils [4–6], and several reports have demonstrated CRP/neutrophil interactions in vivo [5] and in vitro [7–12]. Binding of CRP to neutrophils [9–11] appears to play a dual role. At concentrations ≤1 μg/ml, CRP elicits neutrophil chemotaxis and phagocytosis, whereas at higher concentrations it can inhibit superoxide production and secretion [12].

To gain more insight into the latter CRP effects, we have attempted to correlate its inhibitory effect on superoxide generation and secretion with two known neutrophil regulatory mechanisms: protein phosphorylation by protein kinases [13], and Ca^{2+} mobilization [14]. We have used as stimulants PMA, which activates PK-C [15], and fMLF, a more relevant physiological stimulant, which acts via specific receptors [16].

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Abbreviations: CRP, C-reactive protein; PMA, phorbol-12-myristate-13-acetate; fMLF, *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine; fura2, 1-[2-(5-carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxy]-2-(2′amino-5′methylphenoxy)ethane-*N,N,N′,N′*-tetraacetic acid; BSA, bovine serum albumin

2. MATERIALS AND METHODS

2.1. *Preparation of C-reactive protein*

CRP was purified from acute-phase sera as described [12].

2.2. *Stimulation of ^{32}P-loaded neutrophils*

Neutrophils (≥95%), isolated from human venous blood as in [12], were suspended in 20 mM Hepes, brought to pH 7.4 with NaOH at 37°C, containing 120 mM NaCl, 5 mM KCl, 0.5 mM $MgSO_4$ and 5 mM glucose (buffer A). Neutrophil suspensions in buffer A with 0.2% BSA (5×10^6/ml) were incubated for 60–90 min at 37°C with carrier-free $H_2{}^{32}PO_4$ (1 mCi/ml) (Radiochemical Centre, Amersham, England).

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After washing twice with buffer A, the neutrophils were resuspended at 1.5×10^6/ml in the same buffer, supplemented with 1 mM $CaCl_2$ [17].

Pre-loaded neutrophils (3×10^6), in a final volume of 0.25 ml, were treated for 2 min with either 10^{-7} M fMLF (Sigma, St. Louis, USA) in the presence of 5 μg/ml of cytochalasin B (Serva, Heidelberg), or 20 ng/ml of PMA (Sigma), or for 5 min with 0.3–100 μg/ml of CRP. In the case of treatments with fMLF or PMA, appropriate controls were run containing cytochalasin B and the stimulant solvent dimethyl sulfoxide (final concentration 0.1%). Alternatively, neutrophils were incubated with CRP (0.3–100 μg/ml) for 5 min and then challenged with either fMLF or PMA for a further 2 min. The reaction was stopped with 0.1 ml of 21% (w/v) trichloroacetic acid and the precipitated proteins were collected by centrifugation and solubilized in Laemmli sample buffer as described [17]. When necessary, pH was restored to 6.8 with a few microliters of concentrated NaOH.

2.3. *Separation of proteins by SDS-PAGE and autoradiography*

After SDS-PAGE, the gels were fixed, stained, dried and subjected to autoradiography as in [17]. The grey level images of bands in autoradiographic films were analyzed automatically by the IBAS 2000 system (Kontron, Zürich). The integrated absorbances (IA) of the images are proportional to the extent of [^{32}P]-phosphorylation [17]. The IAs of [^{32}P]phosphoproteins of cells, stimulated either with PMA or fMLF with or without pre-treatment with 100 μg/ml CRP, were divided by the values obtained from unstimulated cells. This provided an estimation of both the enhancement in protein phosphorylation caused by neutrophil stimulation and the effects of pre-treatment with CRP on this process.

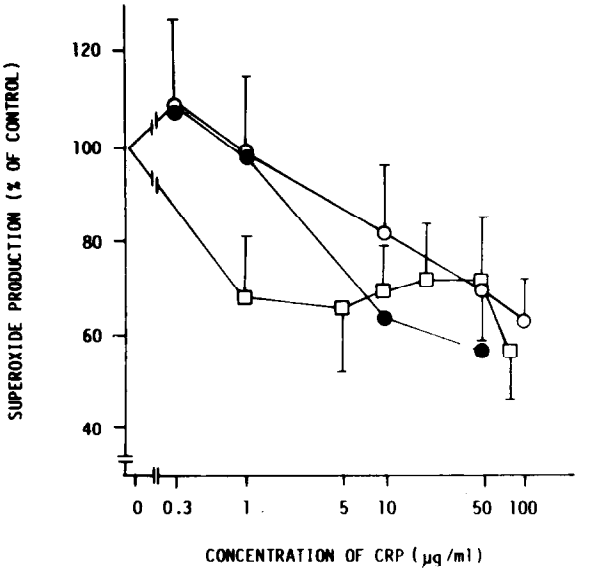

Fig.1. Effect of CRP on superoxide production. Neutrophils were preincubated with CRP for 5 min, then subsequently challenged with either 20 ng/ml PMA (□—□) or 10^{-7} M fMLF (○—○), for a further 5 min. Control values (100%, corresponding to 4.5 ± 0.3 nmol superoxide/10^6 neutrophils per min) refer to cells treated with stimulants in the absence of CRP. Data are means ± SE (PMA, 8 experiments; fMLF, 5 experiments). Cells were also preincubated with BSA (1 mg/ml) and subsequently challenged with fMLF (●—●); mean of two experiments.

2.4. *Superoxide production and secretion*

Superoxide production and secretion of vitamin B_{12}-binding protein were determined as previously described [12].

2.5. *Measurement of intracellular Ca^{2+}*

Measurement of cytosolic Ca^{2+} was performed with the Ca^{2+} fluorescent probe fura2 [18] as previously described [19].

3. RESULTS

3.1. *Superoxide production and secretion*

Preincubation of neutrophils with increasing amounts of CRP (0.3–100 μg/ml) for 5 or 10 min, followed by exposure to either fMLF (10^{-7} M) or PMA (20 ng/ml) resulted in an increasing inhibition of superoxide production up to 40–50% (fig.1). Addition of CRP (100 μg/ml) together with, or following the stimulant resulted in lower inhibition, <20%. The presence of BSA did not significantly alter the inhibitory effect of CRP

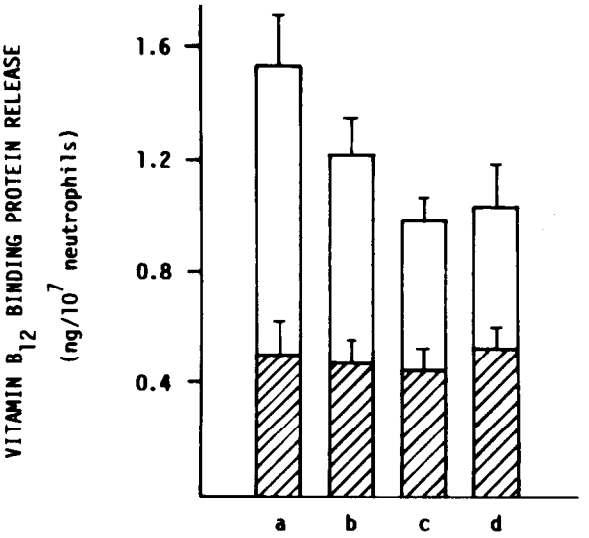

Fig.2. Effect of CRP on secretion of vitamin B_{12}-binding protein. Neutrophils were preincubated with CRP (5–10 min) and then subsequently challenged with fMLF 10^{-7} M (15 min). (a) fMLF alone; (b–d) 1, 10 or 50 μg/ml CRP, followed by fMLF. Hatched areas: no addition of fMLF. Data are means of at least three experiments ± SE.

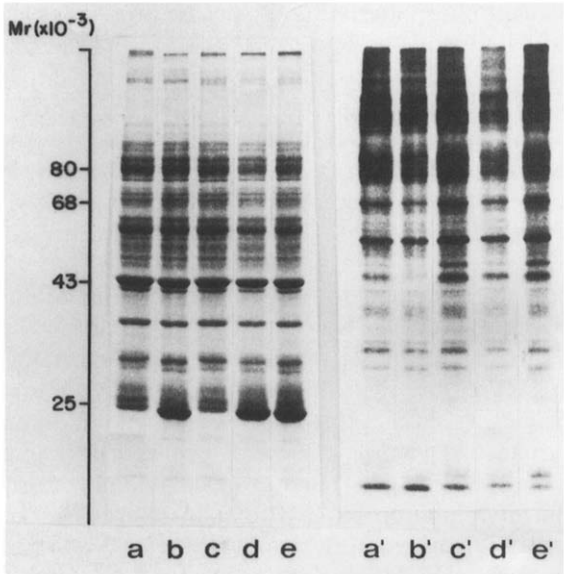

Fig.3. Protein phosphorylation in ^{32}P-labelled human neutrophils treated with PMA (20 ng/ml) and CRP (100 μg/ml) as characterized by SDS-PAGE (10% acrylamide). (a–e) Coomassie blue stain, (a′–e′) autoradiograph. Lanes: (a,a′) control, 2 min (7 min incubation was similar); (b,b′) CRP, 5 min; (c,c′) PMA, 2 min; (d,d′) preincubation with CRP for 5 min, followed by addition of PMA for 2 min; (e,e′) PMA and CRP incubated together with cells for 7 min.

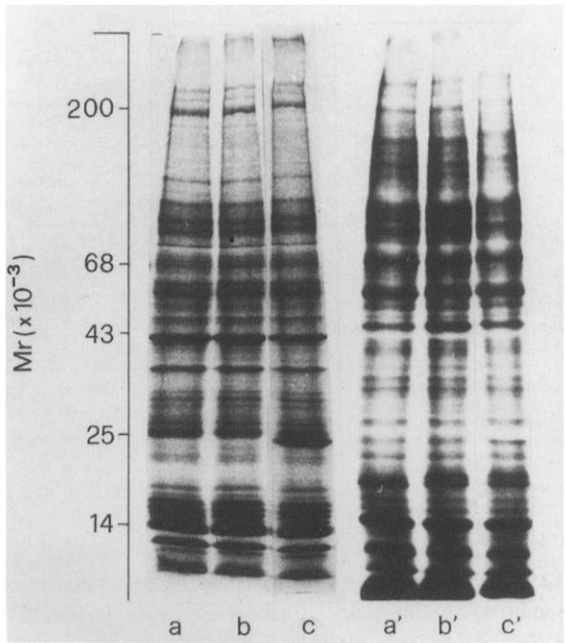

Fig.4. Protein phosphorylation in ^{32}P-labelled human neutrophils treated with fMLF (10^{-7} M) and CRP (100 μg/ml) as characterized by SDS-PAGE (8–18% linear gradient of acrylamide). (a–c) Coomassie blue stain, (a′–c′) autoradiograph. Lanes: (a,a′) control, 2 min (7 min incubation was similar); (b,b′) fMLF, 2 min; (c,c′) preincubation with CRP for 5 min, followed by addition of fMLF for 2 min.

(fig.1), suggesting that an excess of another serum protein does not block the action of CRP. Preincubation of neutrophils with CRP also inhibited the secretion of vitamin B_{12}-binding protein induced by fMLF (fig.2).

3.2. *Phosphorylation of neutrophil proteins*

Activation of neutrophils with PMA or fMLF increased the phosphorylation of several proteins, most notably the 66, 47 and 43 kDa bands, as compared to the absence of stimulant (fig.3, lane c vs a; fig.4, lane b vs a; and table 1). Cytochalasin B, included in the fMLF reaction medium, had no effect on protein phosphorylation per se. CRP (100 μg/ml) alone, as compared to the control, inhibited phosphorylation of at least the 66, 47 and 43 kDa proteins (fig.3, lane b vs a).

Preincubation for 2 min with CRP (100 μg/ml) for 5 min, followed by a challenge with either PMA (fig.3, lane d) or fMLF (fig.4, lane c), resulted in a marked inhibition of the stimulant-enhanced phosphorylation of several proteins (data of Coomassie-stained gels exclude the possibility that this decrease is due to reduced protein loading on gels). These included the proteins of approx. 85, 66, 54, 47 and 43 kDa (table 1). CRP added together (fig.3, lane f) or 2 min after (not shown) the stimulant had only a negligible effect on the stimulant-enhanced phosphorylation.

3.3. *Phosphorylation of CRP*

In some of the above autoradiographs a phosphorylated protein band of 24 kDa was observed, which was superimposed on the CRP band in the Coomassie-stained gels. This suggests that CRP was exposed to a kinase when incubated with the neutrophils. Binding assays performed at 37°C have shown an extremely fast binding and internalization of CRP [11]. In a cell-free system, phosphorylation of CRP was achieved only in the presence of activated PK-C, but not of cAMP-dependent protein kinase (unpublished).

Table 1

Effect of a CRP pretreatment on the phosphorylation of some proteins in neutrophils stimulated with PMA or fMLF

Protein	Neutrophil treatment			
	PMA		fMLF	
	− CRP	+ CRP	− CRP	+ CRP
85 kDa	1.92 ± 0.33	1.07 ± 0.17	1.70; 2.53	1.41; 1.33
66 kDa	3.03 ± 0.44	1.26 ± 0.26	2.73; 3.06	1.40; 1.75
54 kDa	1.78 ± 0.10	0.86 ± 0.15	1.70; 3.74	1.04; 1.96
47 kDa	4.13 ± 1.33	1.77 ± 0.12	2.61; 4.67	1.91; 1.80
43 kDa	6.35 ± 1.42	3.28 ± 0.60	7.75; 4.43	2.27; 1.98

Experimental conditions as in figs 3,4. Numbers are ratios of integrated absorbances of images corresponding to the [^{32}P]phosphorylated protein bands in autoradiographic films (stimulated/resting cells; see section 2), and are means ± SE (5 experiments) for PMA and two separate experiments for fMLF

3.4. *CRP and mobilization of intracellular Ca^{2+}*

Incubation of neutrophils with CRP neither affected the basal level of intracellular Ca^{2+} nor, upon preincubation, inhibited the kinetics and extent of the fMLF induced increase in intracellular Ca^{2+} (not shown).

4. DISCUSSION

Neutrophil functional responses are largely inhibited in cells obtained from synovial fluid, thermal injury sites, and from experimentally inflammatory sites [20–22]. The rate of superoxide production by exudate neutrophils is decreased when compared with peripheral blood neutrophils from the same donor [23]. In all these situations, CRP would be expected to be elevated at the site of neutrophil accumulation, its level corresponding to the degree of inflammation. The 'defects' observed in neutrophil function may thus result from exposure and interaction of the cells with CRP at the inflammatory site, as also suggested by the effects of the in vitro exposure of neutrophils to CRP before stimulation with fMLF (here described) or PMA or concanavalin A [12].

In neutrophils as well as in many other cell types, the consequences of ligand-receptor interactions have been shown to result mainly in an increased turnover of phosphatidylinositol and possibly phosphatidylcholine, changes in intracellular Ca^{2+} and activation of protein kinases [13,14,17,19, 24–30]. In particular, activation of PK-C and subsequent phosphorylation of specific proteins, such as the cytosolic proteins of 43 and 46–47 kDa, has been suggested as an important regulatory mechanism in neutrophil activation [13,17,24,27, 28]. This enzyme is normally localized in the cytosol and becomes fully active after translocation to the plasma membrane [27], where phosphatidylserine, diacylglycerol and Ca^{2+} synergise to activate the translocated enzyme.

In the attempt to understand the molecular mechanism(s) by which CRP may inhibit the neutrophil response, we have exposed neutrophils to CRP before the stimulants PMA or fMLF and have observed that this causes a marked inhibition of phosphorylation of several proteins including those of 85, 66, 54, 47 and 43 kDa. Actually the extent of inhibition by CRP of phosphorylation of some of these proteins, notably the PK-C substrate of 47 kDa [17,24], appears to exceed the degree of inhibition of neutrophil functions. This, however, is in accordance with the concept that neutrophil activation may involve not only the activation of PK-C, but also of other pathways dependent on Ca^{2+} [13]. In this connection, it is important to point out that CRP does not affect the kinetics and extent of cytosolic Ca^{2+} rise in neutrophils activated with fMLF.

The observed inhibition of phosphorylation of some proteins, including those whose phosphorylation correlates with neutrophil activation [13,17, 24,25,27], may suggest a mode of action of CRP. One possibility might be that this protein slows down the turnover of diacylglycerol-generating phospholipids [30], also in the absence of effects in the calcium rise [31]. CRP inhibition of platelet aggregation has actually been shown to be accompanied by an inhibition of arachidonic acid release from both phosphatidylinositol and phosphatidylcholine, suggesting that phospholipases are inhibited [32]. Furthermore, we have demonstrated that in neutrophils, CRP increases cAMP concentration [12], which under some circumstances may inhibit phospholipase C [33]. This postulated mechanism, however, does not apply to the inhibition by CRP of protein phosphorylation in neutrophils treated with PMA, which directly activates PK-C [15] without the concurrence of phosphatidylinositol breakdown. An alternative target of CRP might thus be a critical step in the membrane translocation/activation of PK-C. Prelimi-

nary experiments, carried out to prove this hypothesis, have actually indicated that preincubation of neutrophils with 100 μg/ml CRP almost totally prevents the PMA-induced translocation of PK-C to the membrane fraction.

An intriguing aspect of the CRP action on neutrophils is the actual cell compartment where CRP interacts with its molecular targets causing the metabolic and functional inhibition. We have previously shown that CRP binding to neutrophils at 37°C and pH 7.4 is saturable, very fast and exhibits a dissociation constant of 3.3×10^{-8} M [11]. The present studies have also demonstrated that CRP, once bound to neutrophils, is available to a protein kinase. Furthermore, ^{125}I-labelled CRP, bound to neutrophils, is not extracted by a non-ionic detergent (Triton X-100), which removes a large fraction of the plasma membrane and all the soluble cytoplasm (unpublished), suggesting that it may be associated to detergent-insoluble cell structures such as the cytoskeleton. The possibility thus exists that CRP acts on neutrophils intracellularly, under conditions where the components of signal transmission and metabolic pathways are fully accessible.

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