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The regulation of superoxide generation and nitric oxide synthesis by C-reactive protein

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SUMMARY

Activated macrophages utilize both reactive oxygen intermediates and reactive oxynitrogen intermediates for defence against microbes. However, simultaneous generation of superoxide (O_2^-) and nitric oxide (NO) could be harmful to host cells due to the production of peroxynitrite, nitrogen dioxide and hydroxyl radicals. Therefore, the regulation of the production of these molecules is critical to host survival. During periods of inflammation or infection, the level of serum C-reactive protein (CRP) increases in many species. Human and rat CRP have been shown to bind and interact with phagocytic cells. Since many of the interactions of CRP involve the binding to the phosphocholine ligand, we studied the role of CRP in O_2^- and NO generation through the modulation of phosphatidylcholine (PC) metabolism in macrophages. This study has shown that, while rat CRP inhibited phorbol myristate acetate- (PMA) induced release of O_2^- by rat macrophages, CRP-treated macrophages released NO in a time- and dose-dependent manner. CRP increased inducible nitric oxide synthase (iNOS) enzyme as well as iNOS mRNA levels in rat macrophages. Tricyclodecan-9-yl-xanthogenate (D609), an inhibitor to PC phospholipase C (PC-PLC), suppressed iNOS induction but enhanced PMA-induced release of O_2^-. These data indicate that an increased level of CRP during periods of inflammation may result in differential regulation of macrophage NADPH oxidase and iNOS activity. Increased hepatic synthesis of CRP may contribute to the mechanism by which phagocytic cells avoid simultaneous O_2^- and NO synthesis, and this could possibly be mediated through the regulation of PC-PLC.

INTRODUCTION

The principal functions of macrophages are the destruction of invading microbial pathogens and the removal of inflammatory debris. Macrophages and other phagocytes utilize various effector responses to achieve this goal. This includes the generation of reactive oxygen intermediates (ROI) and reactive oxynitrogen intermediates (RONI). However, both ROI and RONI are potentially toxic to the host, and the regulation of the generation of these molecules is critical to the host survival.[1] While superoxide anion (O_2^-) is the precursor of ROI such as hydrogen peroxide (H_2O_2) and the hydroxyl radical ($HO^{\cdot}$),[2] biosynthesis of nitric oxide (NO) may lead to the production of RONI such as nitrosonium (NO^+), nitroxyl (NO^-) ions, nitrogen dioxide (NO_2), peroxynitrite (ONOO-) and S-nitrosothiols.[3] O_2^- is produced when respiratory burst oxidase (NADPH oxidase) is activated by stimuli such as microbial pathogens, *N*-formyl-methionyl-leucyl-phenylalanine (FMLP) or phorbol myristate acetate (PMA). O_2^- is generated mainly by macrophages, monocytes and polymorphonuclear cells (PMN) which are expendable cells involved in host defence.[1] In contrast, NO is generated by almost all nucleated cells from L-arginine by three distinct isoforms of nitric oxide synthase which include constitutive low output endothelial and neuronal isoforms (eNOS and nNOS) and an inducible high output isoform (iNOS).[4] The iNOS is induced when cells are stimulated by combinations of cytokines and bacterial products like lipopolysaccharide.[4] Macrophages are capable of producing copious amounts of O_2^- and NO when triggered by appropriate stimuli. During the period of immunological challenge, pathways leading to NO as well as O_2^- can potentially be activated. However, simultaneous generation of O_2^- and NO can lead to the production of $ONOO^-$ and $HO^{\cdot}$ which can cause considerable damage to microbial pathogens as well as to host cells.[5–7]

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Abbreviations: CRP, C-reactive protein; IFN-γ, interferon-γ; NOS, nitric oxide synthase; O_2^-, superoxide; PC, phosphatidylcholine; PC-PLC, phosphatidylcholine-specific phospholipase C; PMA, phorbol myristate acetate.

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Periods of immunological activation are also marked by an inflammatory response aimed at preventing tissue damage and limiting tissue injury. A series of complex reactions that occur during this period constitute the acute-phase response.[8,9] An important systemic reaction during this period is the increased synthesis of acute-phase proteins by the liver.[9] C-reactive protein (CRP) is a prototypical acute-phase protein, usually present in trace amounts in the serum, which increases several thousand-fold during infection or trauma. CRP, a member of the pentraxin family of proteins, has been identified in many mammalian and certain invertebrate species, and has retained the unique property to bind to phosphocholine ligand throughout evolution.[10,11] Human CRP is made up of five non-covalently linked identical monomers, each 23 017 MW consisting of 206 amino acid residues[11] while rat CRP consists of five identical monomers of which two form a dimer linked by interchain disulphide bonds between Cys-208 and Cys-209.[12] Unlike human CRP, rat CRP has two potential glycosylation sites (Asn-128 and Asn-147) of which only one (Asn-128) is glycosylated and has an extra C-terminal heptapeptide (Leu-205 to Ser-211).[12,13] Rat CRP is also present in normal serum in significant levels (0·3–0·5 mg/ml) and increases two- to threefold during infection.[12] CRP is known to interact with the cells of the immune system by different modes. CRP functions as an opsonin;[14] it activates the classical complement pathway by binding to phosphocholine-linked substrates,[15] and induces tumoricidal activity in monocytes and macrophages.[16] Human CRP has also been reported to influence the generation of the O_2^-.[16] A specific receptor for CRP on human neutrophils and monocytes has also been recognized.[14,17,18] Interaction of CRP with leucocytes suggests that CRP may have a role in modulating immune responses. However, the underlying mechanism by which CRP may regulate the activities of these cells remains unclear. We studied the role of rat CRP in PMA-stimulated O_2^- production and iNOS enzyme induction in rat peritoneal macrophages. Since many interactions of CRP involved binding to the phosphocholine ligand, we also assessed the role of phosphatidylcholine-(PC) specific phospholipase C (PC-PLC) in mediating the responses to CRP. Results of this study may provide a basis for the role of CRP in immunopathological conditions.

MATERIALS AND METHODS

Animals and reagents

Male Sprague–Dawley rats (175–200 g) were obtained from Charles River Canada (La Prairie, Quebec), *N*-(1-naphthyl) ethylenediamine hydrochloride, sulphanilamide, cytochrome c and PMA were from Sigma Chemical Company (St. Louis, MO), and D609 was from CalBiochem (La Jolla, CA). Dulbecco's phosphate-buffered saline (DPBS), RPMI-1640 medium, penicillin, streptomycin and trypan blue were from Gibco BRL (Burlington, Ont.). Endotoxin-tested fetal calf serum (FCS) was from Immuno Corp. (Montreal, Canada). Thioglycollate broth was from Difco (Detroit, MI). All other reagents and chemicals were of analytical grade.

Isolation of CRP

Rat CRP was isolated from normal rat serum using a Sepharose–phenylphosphorylcholine affinity adsorbant column as previously described.[19] In some instances, *p*-aminophenyl phosphocholine-conjugated agarose beads (Pierce Chemical Co., Rockford, IL) were also used. Briefly, 10–15 ml of normal rat serum was passed through a column containing *p*-aminophenyl phosphocholine affinity adsorbant, equilibrated with buffer containing 10 mM Tris (pH 7·4) 150 mM NaCl and 2 mM Ca^{2+}. The column was washed extensively and the bound protein was eluted with 8 mM phosphocholine. Eluted protein fractions were dialysed against buffer containing 10 mM Tris (pH 7·4) and 150 mM NaCl. Dialysed fractions were reapplied to a fresh column of *p*-aminophenyl phosphocholine-conjugated agarose and the process was repeated. Serum from inflamed New Zealand White rabbits injected with turpentine (0·5 ml/kg body weight) was used to isolate rabbit CRP according to the same procedure. Human CRP was purchased from CalBiochem. Purity of the protein was routinely checked by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and high-performance liquid chromatography (HPLC; Perkin Elmer series 4) using a gel filtration column (Beckman, UltraSpherogel, 2000 SEC) as described[20] and was found to be more than 95% pure. Purified CRP was tested for endotoxin (*Limulus* amoebocyte lysate assay, Sigma) and found to be below the detectable level of 0·03 EU/ml. Random batches of CRP were also passed through a column containing polymyxin agarose (Sigma) for removal of all traces of lipopolysaccharide (LPS).

Cell culture

Rat peritoneal macrophages were obtained by injecting rats intraperitoneally with 5 ml of sterile 4% thioglycollate broth. Rats were killed by cervical dislocation 72 hr later and macrophages were harvested by peritoneal lavage with DPBS without Ca^{2+} and Mg^{2+} as previously described.[21] The cells were collected by centrifugation (10 min at 400 *g*) of lavage, and washed once with DPBS and suspended in RPMI-1640 medium containing 10% v/v FCS and 1% penicillin and streptomycin solution. Cell viability was monitored by trypan blue exclusion test. Macrophages (1×10^6 cells) were plated in 24-well plates (Falcon) and incubated for 2 hr at 37° in a humidified 5% CO_2 incubator. The plates were then washed with RPMI-1640 three times, fresh medium containing 10% FCS and antibiotics was added and they were incubated for a further 24 hr. The plates were then washed to remove non-adherent cells, the medium was replaced and CRP and/or other reagents were added. The plates were then incubated for various time periods ranging from 0 to 72 hr. Throughout the study, rat CRP was used at concentrations ranging from 20 to 300 μg/ml, as specified.

Superoxide generation

Adherent macrophages were washed twice with DPBS. Cells were incubated at 37° with 80 μM cytochrome c, CRP (50–300 μg/ml) and other agents in Hanks' balanced salt solution (HBSS) to a final volume of 1 ml. PMA (1 μg/ml) was added to trigger superoxide generation. Reaction was stopped by the addition of 2 μg of superoxide dismutase and placing the plates on ice. An aliquot of the medium was transferred to ice-cold PBS, and reduction of cytochrome c was assessed in the cell-free supernatants by measuring the optical density at 550 nm. Superoxide dismutable cytochrome c was calculated by using an extinction coefficient of 21·1 M^{-1} cm^{-1} for cytochrome c. O_2^- was also generated in a

cell-free system containing xanthine oxidase, (Sigma), 10 mU/ml, and acetaldehyde (BDH, Dartmouth, NS, Canada) 20 mM in the presence of cytochrome c (80 μM) and various concentrations of CRP for 5 min at 37° as described.[17,18]

Nitrite determination

Nitrite determination was done by mixing 200 μl of the cell culture fluid with an equal volume of Griess reagent (1% sulphanilamide, 0·1% naphthyl ethylenediamine dihydrochloride, 25% H_3PO_4) and incubating for 10 min at room temperature. Absorbance was read at 543 nm against a standard curve using sodium nitrite.[22]

Cell labelling

Rat peritoneal macrophages were grown overnight in 6-well culture plates (Falcon). Non-adherent cells were removed by washing the cells twice with RPMI-1640 medium. Cell labelling and phospholipase assays were performed essentially as described by Schutz *et al.* with slight modifications.[23] Cells were radiolabelled for 24 hr with 0·5 μCi methyl [^{14}C]choline in medium containing 0·5% FCS. Prior to stimulation, cells were washed three times with DPBS and incubated with medium containing 0·5% FCS for 2 hr. Cells were again washed three times with DPBS and were stimulated with CRP in medium containing 1% bovine serum albumin (BSA). The reaction was stopped by adding ice-cold methanol followed by lipid extraction[24] and phase separation. Water-soluble choline metabolites were resolved by drying the sample under nitrogen and performing thin-layer chromatography in a solvent system containing methanol/0·9% NaCl/ammonium hydroxide (100:100:2).

Western blot

Cell monolayers were grown in 25-ml flasks and treated with various agents and incubated as described. The monolayers were rinsed rapidly with ice-cold DPBS. Boiling lysis buffer (125 mM Tris, pH 6·8, 2% SDS, 5% glycerol, 0·003% bromophenol blue and 1% mercaptoethanol) was added (1 ml/flask) and cells were scraped, transferred into microfuge tubes and boiled for an additional 5 min. The sample was sonicated for 10 seconds, and the samples were centrifuged in a microfuge to remove any insoluble material. Protein concentration was measured by the bicinchoninic acid (BCA) method (Pierce, Rockford, IL). Macrophage lysate was separated on 7·5% reducing SDS–PAGE and transferred in 20% methanol, 25 mM Tris, 192 mM glycine (pH 8·3) to 0·2-μm pore size PVDF membranes (Bio-Rad Laboratories, Richmond, CA). The membranes were blocked with 3% BSA in Tris-buffered saline (20 mM Tris, pH 7·5, 500 mM NaCl) and incubated overnight with rabbit anti-iNOS polyclonal antibody (Transduction Laboratories, Lexington, KY), 1:3000 dilution in Tween TBS (20 mM Tris, pH 7·5, 500 mM NaCl, 1% BSA, 0·05% Tween), washed three times in Tween TBS and incubated with goat anti-rabbit IgG (1:2500 dilution in Tween TBS) conjugated to alkaline phosphatase (Bio-Rad) for 2 hr. The membranes were then washed and developed in carbonate buffer (0·1 M $NaHCO_3$, 10 mM $MgCl_2$, pH 9·8) containing 5-bromo-4-chloro-3-indoyl phosphate (0·15%) and nitroblue tetrazolium (0·3%).

Northern blot

Total RNA was isolated by a rapid guanidinium isothiocyanate method as previously described.[25] RNA was denatured in formamide (50%)/formaldehyde (6·5%) sample buffer at 65° for 15 min and separated on formaldehyde (1·8%)/agarose (0·8%) and transferred to nylon (S & S Nytran) membranes (Schleicher & Schuell, Keene, NH). RNA was fixed by drying the membranes and baking for 2 hr at 80° in a vacuum oven. The membranes were then prehybridized, and hybridized with cDNA probe specific for murine iNOS (1·8-kilobase fragment of mouse macrophage iNOS gene, Cayman Chemical Company, Ann Arbor, MI) which was random-primer-labelled with [α-^{32}P]dCTP (6000 Ci/mM, 10 mCi/mi; Dupont, Boston, MA) using the MegaPrime labelling kit (Amersham Life Sciences, Oakville, Ont., Canada) according to the manufacturers' instructions. The membranes were simultaneously hybridized with a random primer-radiolabelled glyceraldehyde-3-phosphate dehydrogenase cDNA probe (Clonetech Laboratories, Palo Alto, CA) to control for equal loading of RNA and washed at high stringency (final wash, 0·1% SSC, 0·1% SDS at 50°). Autoradiography was done by exposure to Kodak XAR film. Autoradiographs were scanned on an LKB 2222-020 enhanced UltroScan XL laser densitometer and mRNA levels were quantified by using a Gelscan XL software(Pharmacia LKB Biotechnology, Baie d'urfé, PQ, Canada).

RESULTS

Effect of CRP on O_2^- generation

Rat peritoneal macrophages produced copious amounts of superoxide when triggered with PMA. This sustained generation of O_2^- by peritoneal macrophages was linear up to 120 min. A significant reduction of O_2^- production was seen when CRP was included in the assay. Inhibition of superoxide generation by CRP was time dependent and was linear up to 2 hr (Fig. 1a). This inhibition by CRP was dose dependent. In the absence of the stimulus, CRP had no effect on the basal ferricytochrome c reduction (Fig. 1b). To test whether CRP acted strictly as a scavenger of O_2^-, the action of superoxide dismutase (SOD) was compared to that of CRP by adding CRP or SOD at various intervals after stimulating cells with PMA and continuing incubation for up to 90 min. It was necessary to add CRP within 30 min of PMA stimulation to cause any significant inhibition of O_2^- synthesis. SOD, on the other hand, decreased the release of O_2^- when added even after 60 min of stimulation. Thus, a lag period was observed with CRP-induced reduction of cytochrome c as compared to the action of SOD (Fig. 2). We also examined the effect of rat CRP on chemically induced O_2^- generation using xanthine oxidase and acetaldehyde. There was a small but significant inhibition of cytochrome reduction in this system when CRP was present at or in excess of 300 μg/ml (Table 1). This indicated that CRP may have some O_2^- scavenging activity when present in excess of 300 μg/ml. To verify that the reduction of cytochrome c was not due to a competing protein we estimated PMA-induced superoxide production in the presence of 200 μg of BSA, human serum IgG, CRP, as well as α-1 acid glycoprotein (α-1AGP), another acute-phase protein. The results indicated that significant inhibition of

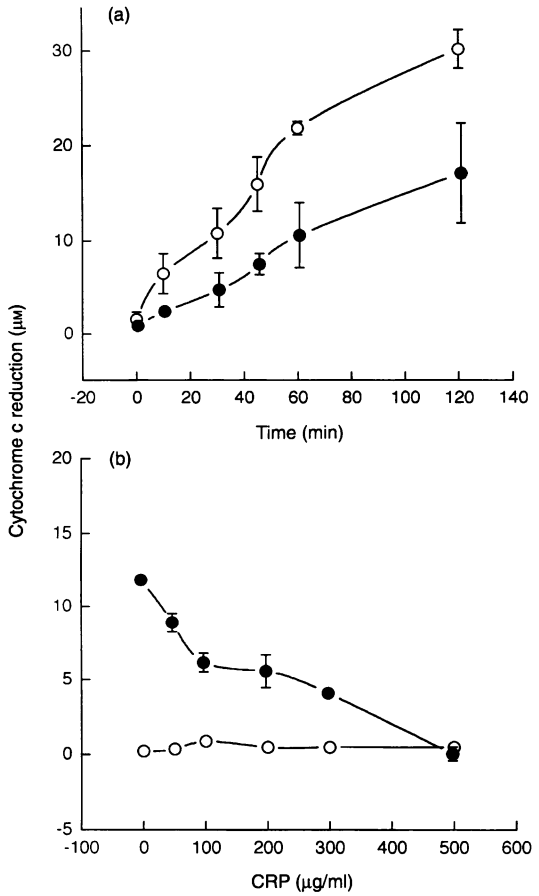

Figure 1. Effect of CRP on of O_2^- generation by PMA-stimulated rat peritoneal macrophages. (a) Inhibition of O_2^- by CRP as a function of time. Cells were incubated with (●) or without (○) CRP (150 μg/ml) for 5 min and then stimulated with PMA (1 μg/ml) to trigger superoxide production for various time intervals. Error bars represent mean ± SD of three to five experiments. (b) Inhibition of O_2^- by CRP as a function of concentration. Cells were preincubated with increasing concentrations of CRP for 5 min before triggering O_2^- production by PMA (●) or left unstimulated (○) and was further incubated for 60 min. Reduction of cytochrome c was used to monitor O_2^- generation. Error bars represent mean ± SD of four to eight experiments.

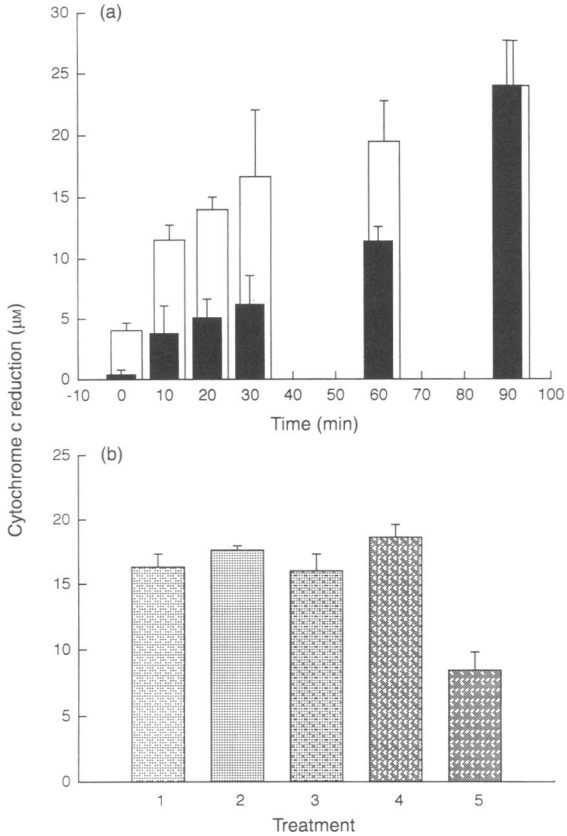

Figure 2. (a) Comparison of CRP and superoxide dismutase on the reduction of PMA-triggered superoxide generation by rat peritoneal macrophages. CRP (300 μg/ml) (□) or superoxide dismutase (2 μg/ml) (■) was added at the time-points indicated. Incubation was continued for 90 min and O_2^- was measured by the reduction of cytochrome c. Data represent mean ± SD of four to six experiments. (b) Effect of various proteins on O_2^- generation in PMA-stimulated rat macrophages. Cells were preincubated 200 μg of protein for 5 min before triggering O_2^- production by PMA and were further incubated for 60 min. Reduction of cytochrome c was used to monitor O_2^- generation. 1, control; 2, BSA; 3, human IgG; 4, α-1 acid glycoprotein; 5, CRP; error bars represent mean ± SD of three to six experiments.

cytochrome c reduction occurred only in the presence of CRP (Fig. 2b).

Induction of NOS by CRP

Rat peritoneal macrophages were tested for the ability of rat CRP to induce NO. Control rat peritoneal macrophages exhibited small but significant baseline NOS activity as measured by nitrite production (Fig. 3a). Basal NO release by rat peritoneal macrophages was variable. This level was increased more than twofold by CRP at a concentration of 20 μg/ml. Treatment of cells by CRP resulted in a time-dependent accumulation of nitrites in the medium which reached a maximum level at 48 hr and was sustained for at least 72 hr (Fig. 3b). The stimulation of nitrite production was detectable at a minimum CRP concentration of 2·5 μg/ml and this reached a maximum level at a concentration of 50 μg/ml cells.

Table 1. Effect of CRP on O_2^- production in a cell-free assay

Treatment	Cytochrome c reduction (nmol/ml)
CRP (0 μg)	14·77 ± 0·663
CRP (75 μg)	14·78 ± 0·736
CRP (150 μg)	14·23 ± 1·19
CRP (300 μg)	13·48 ± 0·775
CRP (450 μg)	12·43 ± 0·792*

O_2^- was produced in a cell-free system consisting of xanthine oxidase and acetaldehyde in the presence of various concentrations of CRP as described in the Materials and Methods. Values represent mean ± SD of net O_2^- produced in six experiments.

*$P < 0.005$ versus assay without CRP by Student's t-test.

CRP induced NOS activity in peritoneal macrophages in a time- and dose-dependent manner (Fig. 3a,b). Western blot analysis was carried out using a polyclonal antibody specific to iNOS to detect NOS protein in the macrophage cell lysates

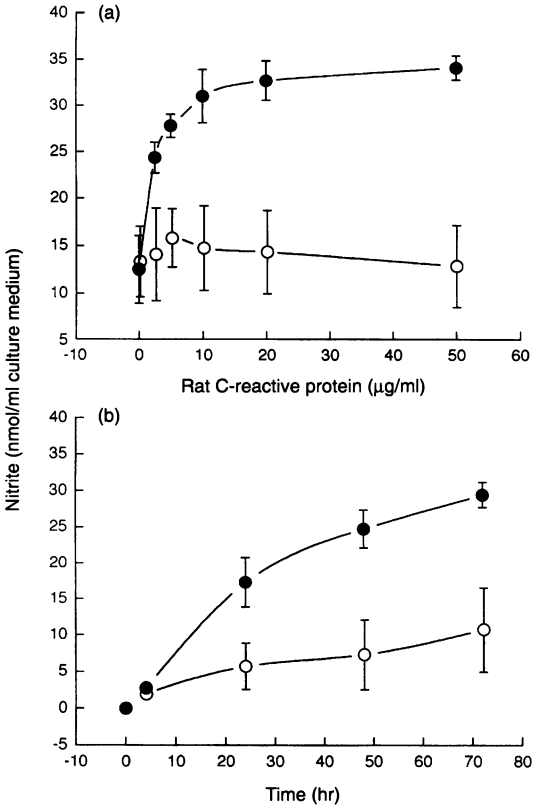

Figure 3. Effect of CRP on generation of NO in macrophages. (a) Effect of increasing concentrations of CRP (●) on NO synthesis by rat peritoneal macrophages. Control cells (○) were treated with an equal volume of buffer (10 mM Tris/150 mM NaCl, pH 7·4). Error bars represent mean ± SD of three to six experiments. (b) Rat peritoneal macrophages were incubated with (●) or without (○) rat CRP (20 μg/ml) for various times. Aliquots of the medium were analysed for nitrite by Greiss reaction. Error bars represent mean ± SD of four to six experiments.

treated with CRP. The Western blot results provided further confirmation of NOS induction by CRP (Fig. 4a). The induction of iNOS was found to be dose dependent by Western blot analysis as well. The iNOS protein was detectable by Western blot with an apparent molecular weight of 130 000, when the cells were treated with as little as 5 μg/ml of CRP (Fig. 4a). The cells were also treated with human and rabbit CRP, and the immunoblot analysis of this showed that these acted similar to rat CRP (Fig. 4b). Northern blot analysis was carried out to test if CRP influenced iNOS mRNA transcription. CRP (10–50 μg/ml) produced an increase in iNOS mRNA levels in rat peritoneal cells (Fig. 5). Laser densitometric analysis of two different blots showed that when normalized for GAPDH mRNA levels, there was a twofold increase in iNOS mRNA levels in cells treated with 50 μg of CRP. The iNOS mRNA appeared as a single band of 4·2 kilobases.

Phosphatidylcholine hydrolysis in rat peritoneal cells

We examined the effect of CRP on PC hydrolysis since CRP is known to bind to phosphocholine, the hydrophilic head group of PC. Thioglycollate-elicited macrophages were labelled with [^{14}C]choline for 24 hr. The cells were made quiescent by incubating in medium containing 0·5% FCS for 2 hr. Cells were then washed three times with PBS and incubated with or without CRP (50 μg/ml) for up to 30 min. The products were extracted by the method of Bligh & Dyer.[24] Analysis of the products on TLC showed the formation of [^{14}C]phosphocholine indicating phospholipase C (PC-PLC) activity. Production of [^{14}C]phosphocholine was rapidly increased following CRP stimulation as compared to untreated cells. This increase in PC hydrolysis in the presence of CRP fell to normal levels in about 30 min after stimulation (Fig. 6). These results showed that CRP activated PC-PLC activity in rat macrophages.

Effect of PC–PLC inhibition on O_2^- generation and NO synthesis

Tricyclodecan-9-yl-xanthogenate (D609), a specific inhibitor of PC-PLC (10 μg/ml), completely inhibited NO synthesis stimulation by CRP at a concentration of 10 μg/ml (Fig. 7a). On the other hand, D609 increased PMA-triggered O_2^- generation by macrophages. D609 had no effect on O_2^- generation on its own in the absence of superoxide triggering agents such as PMA. Addition of PC-PLC derived from *Bacillus cereus* (0·05 units/ml) also decreased PMA-triggered O_2^- generation. This decrease in O_2^- generation by PC-PLC was reversed by D609 (Fig. 7b).

DISCUSSION

In this study we have shown that CRP suppresses O_2^- production and induces NOS in rat macrophages. The data presented here also suggest that the modulation of macrophage function by CRP may involve PC hydrolysis. There have been conflicting reports on the effect of human CRP on O_2^- generation. While there have been reports that human CRP decreases the production of O_2^- by activated neutrophils and monocyte/macrophages,[17,26] there are also reports of increased O_2^- and H_2O_2 production after prolonged exposure to CRP.[16,27] Peptides generated from human CRP by activated macrophages have also been shown to suppress O_2^-.[28] Our results show that rat CRP, which shares 65% homology with human CRP, inhibits O_2^- generation by PMA-stimulated rat peritoneal macrophages in a time- and dose-dependent manner. This inhibition was specific to CRP since similar concentrations of various proteins such as BSA, human IgG or α-1 AGP, failed to inhibit cytochrome c reduction in PMA-stimulated rat macrophages. It is unlikely that the decrease in superoxide generation was due to a change in Ca^{2+} levels, since CRP was eluted from a phosphocholine agarose affinity column with an excess of phosphocholine, and not by EDTA before dialysing extensively to remove the bound phosphocholine. It has been suggested that CRP may act as a scavenger of the O_2^- radical.[17] Our data show that when CRP was added at various intervals after PMA stimulation, significant reduction of O_2^- occurred only when CRP was added within 30 min after the addition of PMA. SOD inhibited cytochrome c reduction significantly at all time-points after the addition of PMA. Moreover, the inhibition of chemically induced O_2^- generation was highly significant only when CRP was present in excess of 300 μg/ml. Buchta *et al.* have shown that human CRP at concentrations as high as 100 μg/ml, did not scavenge O_2^- in a cell-free O_2^-

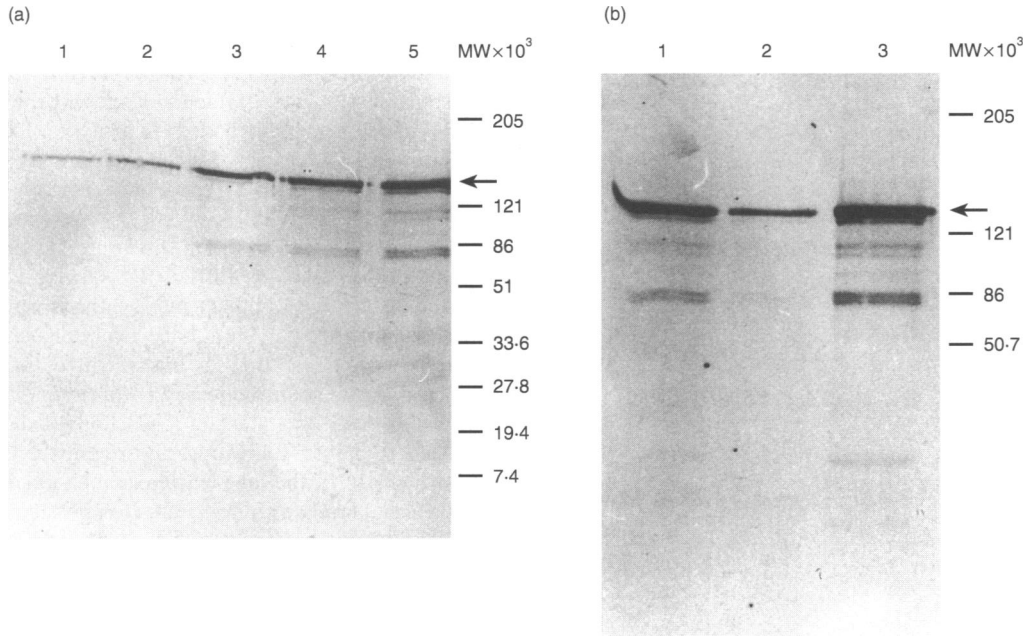

Figure 4. Western blot analysis of iNOS protein in macrophage cells treated with rat and human CRP. (a) Rat peritoneal macrophages treated for 18 hr with different doses of rat CRP; lane 1, untreated; lanes 2, 3, 4 and 5, rat CRP at 5, 10, 20, or 50 μg/ml, respectively. (b) Rat macrophages stimulated with CRP (50 μg/ml) from different species; lane 1, rat CRP; lane 2, rabbit CRP, lane 3, human CRP. Arrow indicates iNOS protein (MW 130 000).

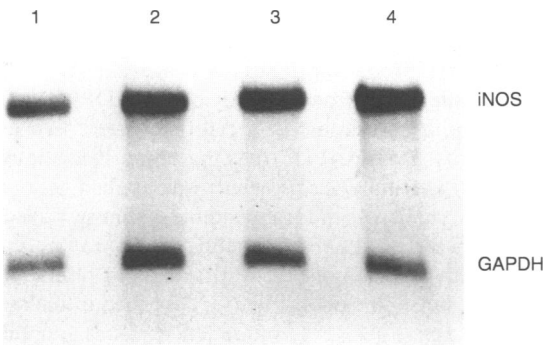

Figure 5. Northern blot analysis of iNOS mRNA (4·2 kilobases) and phosphoglyceraldehyde dehydrogenase mRNA (1·3 kilobases) from macrophage cells treated with rat and human CRP. Rat macrophages treated with rat CRP; lane 1, untreated; lanes 2, 3 and 4, rat CRP at 10, 20 or 50 μg/ml, respectively.

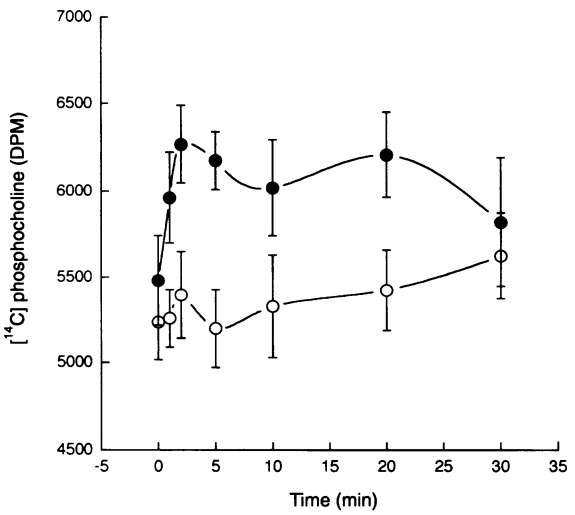

Figure 6. PC hydrolysis in rat macrophages stimulated with CRP. Cells labelled with ^{14}C-labelled choline for 48 hr. Cells were washed and treated with CRP for various times. Hydrolysis of PC was monitored by monitoring the release of water soluble phosphocholine counts. CRP (●), control (○); data represent mean ± SD of three experiments.

generation system.[26] This indicates that the CRP may decrease the level of superoxide produced by stimulated macrophages by direct cell inhibition as well as by scavenging of free radicals.[17,26] During inflammation and tissue injury CRP may demonstrate some antioxidant properties and thus limit the effects of free radicals. CRP has also been shown to decrease PMA-induced phosphorylation of several proteins including those of 85 000, 66 000, 54 000, 47 000 and 43 000 MW.[29] Our data are consistent with the view that CRP acts on neutrophils and macrophages intracellularly where the components of signal transduction and metabolic pathways are fully accessible.[20,29]

In addition to the suppression of O_2^-, CRP also induces a dose-dependent increase in the synthesis of NO in rat peritoneal cells. These cells exhibited small, but significant baseline NOS activity. Baseline NOS activity in rat peritoneal macrophages may be due to partial activation during the isolation of the cells or due to thioglycollate treatment of the rats. Similar observations were noted in thioglycollate-elicited mouse macrophages.[30] We used thioglycollate-elicited macrophages for NOS and O_2^- estimation since resident macrophages produced little or no superoxide when stimulated with PMA. This observation is consistent with previous reports.[31] The induction of iNOS protein by CRP was confirmed by both

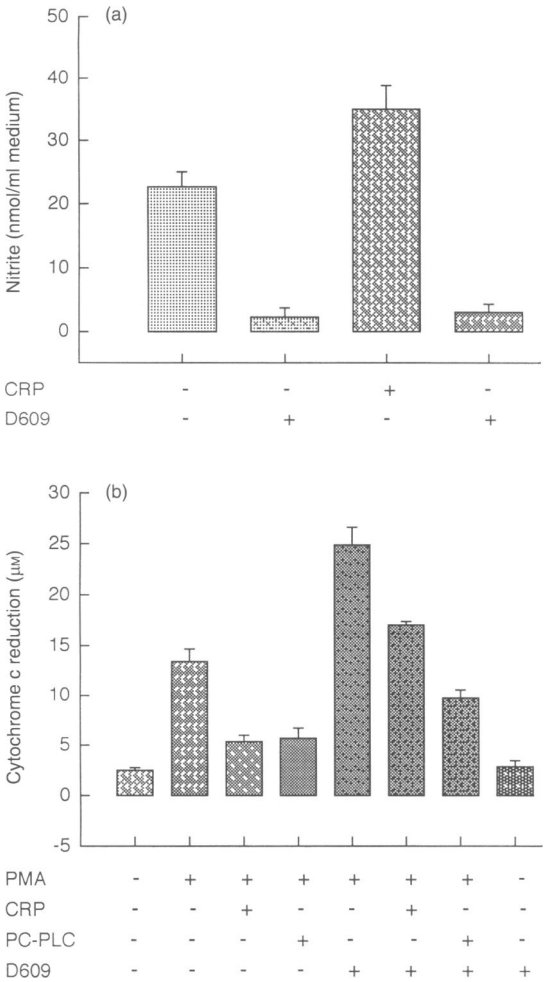

Figure 7. (a) Inhibition of NO synthesis by D609. Rat macrophages were pretreated with D609 (20 μg/ml) for 20 min before the addition of CRP (20 μg/ml). Presence (+) or absence (−) of agents used is shown. Data represent mean ± SD of four experiments. (b) Stimulation of superoxide generation by rat peritoneal macrophages by D609. Rat macrophages were stimulated by PMA (1 μg/ml) and O_2^- production was measured in the presence of CRP (150 μg/ml); PC-PLC (0·05 U/ml); D609 (20 μg/ml). Presence (+) or absence (−) of agents used is shown as indicated. Data represent mean ± SD of four experiments.

Western and Northern blotting. CRP derived from other species, such as human and rabbit, also induced iNOS activity in rat peritoneal macrophages. This was not surprising considering the high degree of homology between human, rabbit and rat CRP. Retro-inverso peptide analogues, derived from CRP and resembling the amino acid sequence of tuftsin, have been shown to induce NO synthesis.[32] It is also of interest to note that both rat and human CRP contain tuftsin-like peptide sequences. Tuftsin, a tetrapeptide (TKPR) derived from the C_H2 domain of IgG by proteases, is a known activator of phagocytic cells and has been implicated as an inducer of NO synthesis.[33,34] Previous work from our laboratory[20] has shown that rat CRP not only bound to rat macrophages but was also internalized and degraded into small peptides. We are currently investigating the nature of these peptides and whether the generation of peptides is a necessary prerequisite for the induction of iNOS in macrophages by CRP.

One of the striking properties of CRP is its affinity for the phosphocholine ligand, which is also the product of PC-PLC catalysed phosphatidylcholine hydrolysis. D609, a specific inhibitor of PC-PLC,[23,35] was shown to inhibit NO synthesis in J774 cells stimulated by lipoteichoic acid or lipopolysaccharide and interferon-γ (IFN-γ)[36,37] The antiviral xanthate compound, D609 has also been shown to be an effective and specific inhibitor of PC-PLC.[23,35,38] Our results have shown that D609 not only inhibits NO synthesis but also increases the secretion of O_2^- in stimulated macrophages. D609 had no effect on O_2^- generation in unstimulated macrophages. We observed a significant increase in the generation of phosphocholine on CRP treatment of [^{14}C]choline-labelled rat peritoneal macrophages, indicating phospholipase C activity. This rapid increase in the labelled phosphocholine fraction fell to near normal levels within 30 min. It is possible that CRP, like growth factors, not only evokes hydrolysis of PC but also activates (re)synthesis of PC resulting in a PC cycle.[38] It is also likely that PMA-sensitive protein kinase C (PKC) isoform(s) may be regulated by PC-derived diacylglycerol (DAG) or other products of PC hydrolysis. One possibility is that membrane translocation/activation of PMA-sensitive PKC may be affected. Addition of bacterial PC-PLC to the cell culture did indeed decrease O_2^- generation and D609 was able to offset this inhibition. Bacterial PC-PLC was previously shown to inhibit O_2^- production by reversibly inhibiting NADPH oxidase in human polymorphonuclear leucocytes.[39] Previous work from our laboratory has shown that CRP hydrolysed dipalmitoyl phosphatidylcholine (DPPC) releasing DAG and phosphocholine in a cell-free assay.[40] To address the possibility that PC-PLC may be co-purified along with CRP, PC-PLC from *Bacillus cereus* was applied to a column containing sepharose–phosphocholine affinity adsorbent. Although bacterial, PC-PLC bound weakly to the column, PC-PLC activity was not detected in column fractions when eluted with 8 mM phosphocholine as described in the methods for purification of CRP (Mookerjea & Hunt, unpublished observations). To our knowledge PC-PLC activity has not been demonstrated in normal rat serum from which rat CRP was isolated. Therefore, we believe that it is unlikely that CRP may be contaminated with PC-PLC. The results presented here indicate a role for PC-PLC activation in the modulation of O_2^- generation as well as NO synthesis. Assuming that D609 causes specific inhibition of PC-PLC as previously reported,[34,35] the inhibition of O_2^- secretion and the stimulation of NO synthesis by CRP is possibly mediated through the activation of PC-PLC. It is also possible that intact membrane PC may act as a steric facilitator in the assembly of the components of NADPH oxidase.[39]

Although 5–20 μg/ml CRP is sufficient to stimulate iNOS synthesis in rat peritoneal macrophages, concentrations of 50 μg/ml or more were required to cause significant inhibition of PMA-stimulated superoxide production. PMA activates the phosphorylation of p47phox and subsequent translocation and assembly of NADPH complex[1] and this involves relatively rapid series of events. Preincubation with CRP may initiate events that may prevent the activation of NADPH complex. Higher concentrations of CRP may be required not only to initiate PC hydrolysis and hitherto unknown events, but also

for some free radical scavenging activity. Activation of iNOS induction by CRP may also involve the initiation of a signalling pathway that may include PC hydrolysis and subsequent events leading to iNOS transcription. Partially activated macrophages are known to express iNOS mRNA on stimulation by a second signal.[1,4] Thioglycollate-elicited macrophages may require far lower concentrations of a second signal, such as CRP, to initiate the cascade of events leading to the prolonged activation of iNOS than that required for the inhibition of O_2^- production triggered by an extremely potent agonist such as PMA.

During the period of bacterial invasion, macrophages are deployed to destroy and remove invading micro-organisms or inflammatory debris. The level of CRP also increases dramatically during periods of immunological challenge.[41] CRP has also been shown to localize at inflammatory sites[42] Macrophages and other phagocytes generate O_2^- as a result of phagocytic triggering, which is the precursor of other ROI including H_2O_2 and hydroxyl radical.[2] Pumping of large concentrations of electrons, such as O_2^-, unaccompanied by protons results in an increase in vacuolar pH, and subsequent activation of neutral proteases which aid in the killing and digestion of bacteria.[43] Although the production of ROI contributes to the bactericidal property of macrophages, oxidation of cellular molecules by ROI can result in severe damage to the host tissue.[5] Macrophages also produce an abundance of NO when activated by an appropriate stimulus. Although both NADPH oxidase and iNOS activity can be induced in macrophages, the secretion of O_2^- and NO does not occur simultaneously and is shown to be independently regulated.[44,45] Ding *et al.*[45] reported that of the 12 cytokines studied, only IFN-γ primed macrophages for increased ROI and RONI production. Monocytes produce O_2^- but as they mature into macrophages NO is employed to mediate cytotoxicity.[44] NO has also been reported to neutralize the cytotoxicity of O_2^- under experimental conditions.[46] Independent regulation averts the potential reaction between O_2^- and NO which forms peroxynitrite ($ONOO^-$)[6] which can produce irreversible damage to both microbes as well as host cells.[6,7] In general cells seem to avoid simultaneous generation of O_2^- and NO. In summary, our results show that CRP differentially regulates NADPH oxidase and iNOS activity in rat peritoneal macrophages. Increased hepatic synthesis and elevation of plasma CRP concentration during periods of inflammatory response may contribute to the mechanism by which immunologically activated cells avoid simultaneous O_2^- and NO biosynthesis.

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