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C-reactive protein (CRP) peptides inactivate enolase in human neutrophils leading to depletion of intracellular ATP and inhibition of superoxide generation

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SUMMARY

The nature and the biochemical mechanism of inhibition of neutrophil membrane-associated oxidative metabolism by two synthetic peptides p77-82 and p201-206 (amino acid sequences Val-Gly-Gly-Ser-Glu-Ile and Lys-Pro-Gln-Leu-Trp-Pro respectively, from the primary amino acid sequence of C-reactive protein) have been ascertained. Preincubating neutrophils for 15 min with 50 μM of p77-82 or p201-206 resulted in superoxide generation by opsonized zymosan stimulated neutrophils being inhibited by $34 \pm 2\%$ ($P < 0{\cdot}005$) and $29 \pm 2\%$ ($P < 0{\cdot}005$) respectively. With a 60-min preincubation period 6·25 μM of p77-82 or p201-206 was effective in inhibiting this superoxide generation by $12 \pm 2\%$ ($P < 0{\cdot}01$) and $10 \pm 1\%$ ($P < 0{\cdot}01$) respectively. Neither peptide inhibited neutrophil arachidonic acid release, transmembrane potential or transductional events preceding superoxide generation. Inhibition of neutrophil functions was found to be due to the ability of each peptide (50 μM) following a 15-min preincubation period to inhibit both neutrophil glycolysis and ATP generation by approximately 30%. The inhibition of ATP generation and glycolysis in neutrophils is attributable to the ability of these peptides to inhibit uncompetitively the glycolytic enzyme enolase. Using purified enolase the relative Ki values for p77-82 and p201-206 were 27 and 19 μM respectively. Inhibition of neutrophil function by the peptides is concluded to be due to effective interference of neutrophil energy metabolism.

INTRODUCTION

We have previously reported that during association of the human acute phase reactant, C-reactive protein (CRP) with neutrophils, low molecular weight CRP peptides are generated.[1] This neutrophil-mediated degradation of CRP was shown to be due to the interaction of CRP with a membrane-associated protease which could be up-regulated by a dose of phorbol 12-myristate 13-acetate (PMA) that is insufficient for the release of azurophil granule enzymes.[1] Many of the pro-inflammatory functions of activated neutrophils were found to be inhibited by the low molecular weight CRP-derived peptides.[2] This mixture of crude bioactive CRP peptides has been shown to contain amongst other peptides three peptides with sequences homologous to the amino acid sequences at positions 77-82, 83-90 and 201-206 of the intact protein.[2] Significant inhibition of neutrophil superoxide production and neutrophil locomotion in response to several stimuli, was demonstrated with synthetic peptides modelled on these three identified sequences.[2] Peptide-mediated alterations in oxidant release from activated neutrophils appeared to be due to a direct inhibitory effect of these synthetic peptides on superoxide production since no oxidant scavenging properties were detected.[2] The present study further characterizes the inhibition of neutrophil superoxide generation by the two most potent of these synthetic peptides, i.e. the peptides with amino acid sequences Val-Gly-Gly-Ser-Glu-Ile and Lys-Pro-Gln-Leu-Trp-Pro (called p77-82 and p201-206 respectively in this study) corresponding to the amino acid sequences at positions 77-82 and 201-206 respectively of the native CRP molecule. In addition the probable biochemical mechanism by which these two peptides alter phagocyte functions has been identified.

Abbreviations: CRP, C-reactive protein; di-*O*-C_5 (3), 3,3[1]-dipentyloxacarbocyanine; LECL, lucigenin (*bis*-*N*-methylacridinium nitrate)-enhanced chemiluminescence; OZ, opsonized zymosan; 2PGA, 2-phosphoglyceric acid; PMA, phorbol 12-mysistate 13-acetate; p70-74, Asp-Ile-Gly-Tyr-Ser; p160-165, Asn-Met-Trp-Asp-Phe-Val; p77-82, synthetic peptide with sequence Val-Gly-Gly-Ser-Glu-Ile; p201-206, synthetic peptide with sequence Lys-Pro-Gln-Leu-Trp-Pro.

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MATERIALS AND METHODS

Chemicals and reagents

These were purchased from the Sigma Chemical Co. (St Louis, MO) with the exception of reagents used for assay of neutrophil glycolytic enzymes and intracellular lactate which were obtained from Boehringer Mannheim (Mannheim, Germany). Radiochemicals were supplied by the New England Nuclear Corp. (Boston, MA).

Synthetic peptides

Synthesis of p77-82 and p201-206 was as described previously.[2] For control purposes two additional synthetic CRP peptides p70-74 (Asp-Ile-Gly-Tyr-Ser) and p160-165 (Asn-Met-Trp-Asp-Phe-Val), generated during neutrophil degradation of CRP[2] were included in the study. These two synthetic peptides correspond to the amino acid sequences at positions 70-74 and 160-165 of the CRP molecule and were not found previously to modulate neutrophil function.[2] All synthetic peptides were partially solubilized in dimethyl sulphoxide (DMSO) and used unless otherwise stated, at a fixed final concentration of 50 μM in the assays described below. This concentration has previously been shown[2] to be the optimal concentration at which p77-82 and p201-206 act individually to significantly inhibit neutrophil oxidative metabolism following a 15-min preincubation period of these peptides with the cells. Appropriate DMSO control systems were included in all assays.

Preparation of crude bioactive peptides (< 14,000 MW)

CRP peptides of less than 14,000 MW were prepared by degrading CRP (200 μg/ml) with PMA-stimulated neutrophils (10×10^6 cells/ml, 10 ng PMA/ml) as described before.[1,2] The trichloroacetic acid (TCA)-soluble peptides following extraction of the TCA, were lyophilized and redissolved in sterile distilled water to a concentration of 1 mg/ml.[1,2] A control was prepared in parallel without CRP as previously described.[1,2]

Neutrophils

These were prepared from preservative-free heparinized venous blood taken from healthy adult volunteers.[1,2] Purified preparations of neutrophils were isolated by means of Hypaque–Ficoll gradients followed by dextran sedimentation and hypotonic lysis of erythrocytes.[1,2] Isolated cells which were 95–98% polymorphonuclear leucocytes, were suspended in indicator-free Hanks' balanced salt solution (HBSS, pH 7·4) for all subsequent incubations.

Measurement of superoxide generation

This was measured using lucigenin (*bis-N*-methylacridinium nitrate)-enhanced chemiluminescence (LECL) as previously described.[1,3] Neutrophils (10^6) were preincubated for 15 min at 37° in 900 μl HBSS containing lucigenin to give a concentration of 0·2 mM in the final assay volume and synthetic peptide at the concentration required in the final assay volume or the appropriate solvent control (DMSO final concentration of 1%). Opsonized zymosan (OZ, 1 mg/ml) was then added (100 μl) and spontaneous and OZ-activated LECL recorded in an LKB Wallac 1251 chemiluminometer (LKB, Turku, Finland). LECL readings were integrated for 5-second intervals and recorded as millivolts/seconds (mV/second). Average peak LECL values are shown. Additional experiments were performed to investigate the effects of the following on peptide-mediated inhibition of the LECL responses of OZ-activated neutrophils (a) various times (15, 30 and 60 min) of preincubation at 37° of cells with the peptides, (b) preincubation of cells with peptides at 37° (15 and 60 min) followed by washing and (c) addition of the peptides to neutrophils 5 min after activation with OZ.

Oxygen consumption

This was measured using a three-channel oxygen electrode (model DW1, Hansatech Ltd, King's Lynn, Norfolk, U.K.). Neutrophils (10^6) were preincubated with the peptides for 15 min at 37° in HBSS followed by addition of OZ. The reduction in P_{O_2} was monitored for a further 20 min after addition of the stimulus.

Measurements of glycolysis, ATP and activity of the glycolytic enzymes

Neutrophils (10^6/ml) were exposed to either the test synthetic peptides, the crude CRP peptides (10 μg), the solvent control or the crude peptide control for 15 min at 37° then concentrated to 1×10^7/ml. Lactate production was used to assay glycolytic activity[4] while intracellular ATP was measured by a luciferin/luciferase chemiluminescence method in cell lysates.[5] The activities of the glycolytic enzymes hexokinase, glucose phosphate isomerase, phosphofructokinase, aldolase, triose phosphate isomerase, glyceraldehyde phosphate dehydrogenase, phosphoglycerate kinase, monophosphoglyceromutase, enolase, pyruvate kinase and lactate dehydrogenase were assayed in the cytoplasmic extracts of the various control and peptide-treated neutrophils as previously described and expressed as percentage of control.[4] The effects of the peptides (0–50 μM) on the activity of purified enolase from rabbit muscle (1 U/assay) were also investigated[4] and expressed as the percentage of control activity in the absence of peptide. In addition the nature of the peptide inhibition on enolase was investigated. An $E_{340} = 6{\cdot}22 \times 10^3$ M/cm for NADH was used to quantitate oxidation of NADH to NAD^+ by enolase in these experiments.

Uptake of radiolabelled glucose by neutrophils

Neutrophils (2×10^6) were co-incubated with 1 μCi of radiolabelled glucose (D-^{3}H-glucose, 15·5 Ci/mmol) with and without the peptides (50 μM) in a final volume 100 μl of HBSS containing 10 μM carrier glucose and incubated for 30 min at 37° on 150 μl of silicone oil in microcentrifuge tubes. Cell-associated and free ^{3}H-glucose were separated by centrifuging the neutrophils through the oil at 12,000 ***g*** for 3 min. After freezing at −70° the neutrophil pellets were sliced from the bottom of each tube and solubilized in Protosol^R (New England Nuclear Corp.). Solubilized pellets and unbound glucose were quantified by liquid scintillation counting and results expressed as nmol glucose/2×10^6 neutrophils.

Measurement of the release of radiolabelled arachidonic acid from neutrophils

Neutrophils ($2{\cdot}5 \times 10^7$/ml) were co-incubated with 5 μCi/ml of radiolabelled arachidonate (5,6,8,9,11,12,14,15-^{3}H(N), 76 Ci/mmol) for 30 min at 37°, then washed twice and resuspended in HBSS. The neutrophils were then incubated for 15 min at 37° with the test peptides or solvent controls prior to the addition of either calcium ionophore (A23187, 2·5 μM) or the riminophenazine compound, clofazimine (10 μg/ml), both of which cause

mobilization of arachidonate from neutrophils.[6,7] After incubation for 5 min at 37° the reactions were terminated by addition of 5 ml of *n*-hexane/isopropanol/concentrated HCl (0·1 M), 300:200:4, v/v/v. Lipids were extracted as previously described.[6] The upper organic phase was separated, retained and dried under a stream of nitrogen. The lipids were dissolved in 100 μl of hexane/isopropanol 3:2, v/v. Aliquots of 10 μl were then spotted onto silica gel 60 F254 precoated thin layer chromatography (TLC) plates (Merck, Darmstadt, Germany). Unlabelled arachidonate standard was added at the origin with the samples to facilitate the visual detection with iodine vapours. The plates were developed in chloroform/acetone 96:4, v/v, the arachidonate spots localized and the silica removed and assayed for radioactivity. These results are expressed as fmol [^{3}H]arachidonate/5×10^7 neutrophils.

Measurement of membrane potential changes

Changes in transmembrane potential were measured using the fluorescent carbocyanine dye 3,3'-dipentyloxacarbocyanine (di-*O*-C_5(3)).[8] The fluorescence was measured with a Hitachi 650-10S fluorescence spectrophotometer (Hitachi Ltd, Tokyo, Japan) equipped with a thermostatted cuvette holder (37°) and magnetic stirrer with excitation and emission wavelengths set at 460 and 510 nm respectively. The neutrophils (3×10^6) were added to a 3-ml cuvette containing 1 μM di-*O*-C_5(3) with or without the peptides or solvent controls in a final volume of 3 ml HBSS. The cells were equilibrated with the probe for 10 min at 37° prior to the addition of PMA (10 ng/ml) and the decrease in fluorescence of di-*O*-C_5(3) followed for 5 min.

Analysis of data

Unless indicated the results of each series of experiments are expressed as either the percentage change or the mean percentage change ±SEM of the corresponding peptide-free control systems. Where appropriate levels of statistical significance were calculated using the paired *t* statistic.

RESULTS

Superoxide generation

A fixed concentration (50 μM) of the peptides p77-82 and p201-206 and a 15-min preincubation period inhibited the LECL responses of OZ-activated neutrophils by 34±5% ($P<0.005$) and 29±2% ($P<0.005$) respectively (Fig. 1). The absolute values for the control system and number of experiments are shown in the legend to Fig. 1.

The kinetics of p201-206 (50 μM)-mediated inhibition of the LECL responses of OZ-activated neutrophils relative to control cells (Fig. 2) indicates that the early LECL response (up to 3 min after stimulus addition) is minimally affected with inhibition becoming increasingly evident with time. A similar result was obtained with p77-82 (data not shown). Addition of the peptides (50 μM) to neutrophils 5 min after addition of OZ caused 18±3% (p77-82) and 15±2% (p201-206) inhibition of peak LECL responses relative to 31±2% and 30±4% when the neutrophils were preincubated (15 min) with the peptides prior to stimulation (three separate experiments).

The extent of inhibition of superoxide generation by OZ-stimulated neutrophils was dependent on the duration of the preincubation period of neutrophils with the peptides at 37°

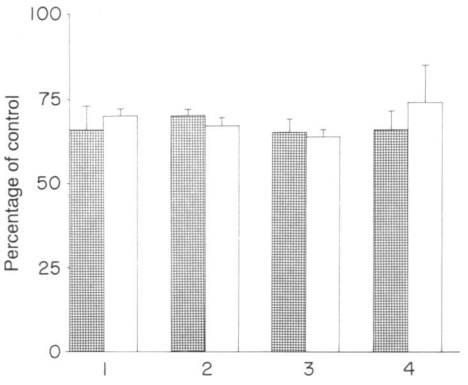

Figure 1. Effects of 50 μM of p77-82 (■) and p201-206 (□) on OZ-activated LECL (1), ATP generation (2), glycolysis (3) and enolase (4) activity in cytoplasmic extracts of peptide-treated neutrophils. The results are expressed as the mean percentage ±SEM of the corresponding control values of six, 14, three and four experiments respectively. The absolute values for the peptide-free control systems for LECL, ATP, glycolysis and enolase were 1645±175 mV/second, 2·4±0·5 nmol ATP/10^7 neutrophils, 45±14 μg lactate/10^7 neutrophils and 3·6±0·2 μM NAD^+/min/10^7 neutrophils.

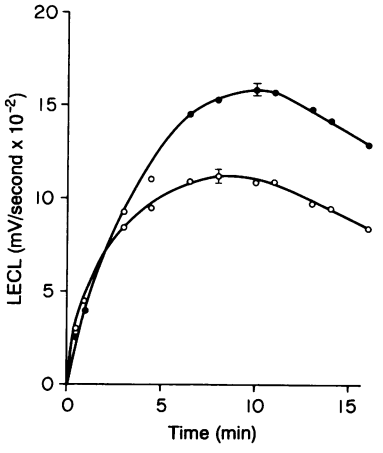

Figure 2. Kinetics of superoxide generation (LECL) by OZ-activated neutrophils co-incubated without (●) and with 50 μm p201-206 (○). Data are from triplicate determinations of a single experiment.

(Table 1). By increasing the preincubation time of the peptides with neutrophils from 15 to 60 min, prior to the addition of OZ, a significant increase in the inhibitory capacity of each peptide as well as significant inhibition using peptide concentrations below 50 μM was achieved (Table 1).

The stability of the inhibition of superoxide generation from OZ-activated neutrophils caused by these peptides, was investigated (three separate experiments) by comparing the OZ-activated LECL response of peptide-treated washed and unwashed neutrophils (Table 2). Increasing the time of preincubation of p77-82 and p201-206 from 15 to 60 min resulted in an increase in the stability of the inhibitory action of each peptide (Table 2).

Table 1. The influence of duration of preincubation of CRP peptides with neutrophils and CRP peptide concentration on the inhibition of the LECL responses of OZ-activated neutrophils

	LECL inhibition (%)†	
	Preincubation time (min)	
Peptide	15	60
p77-82		
50 μM	34±2*	52±1*
25 μM	15±3*	34±2*
12·5 μM	6±1	23±1*
6·25 μM	4±1	12±2**
p201-206		
50 μM	31±2*	65±1*
25 μM	10±1**	30±2*
12·5 μM	8±3	19±1**
6·25 μM	7±1	10±1**

Peak LECL readings 10 min after addition of the stimulus have been expressed as the mean percentage change ±SEM of the peptide-free control system. The absolute value for the peptide-free control system was 1275±109 mV/second. The data for each peptide are from three separate experiments. *$P<0.005$; **$P<0.01$.

Table 2. Stability of CRP peptide-mediated inhibition of the LECL response from OZ-activated neutrophils†

		LECL inhibition (%)	
Peptide	Preincubation time (min)	Not washed	Washed
p77-82			
50 μM	15	28±4*	6±2
50 μM	60	57±1*	27±2*
p201-206			
50 μM	15	30±3*	10±2**
50 μM	60	69±2*	26±3*

Neutrophils were preincubated with the CRP peptides for the indicated time then either immediately activated or washed and then activated with OZ. Peak LECL readings 10 min after addition of the stimulus have been expressed as the mean percentage change ±SEM of the peptide-free control system. The absolute value for the peptide-free control was 1483±112 mV/second. The data for each peptide are from three separate experiments. *$P<0.005$; **$P<0.01$.

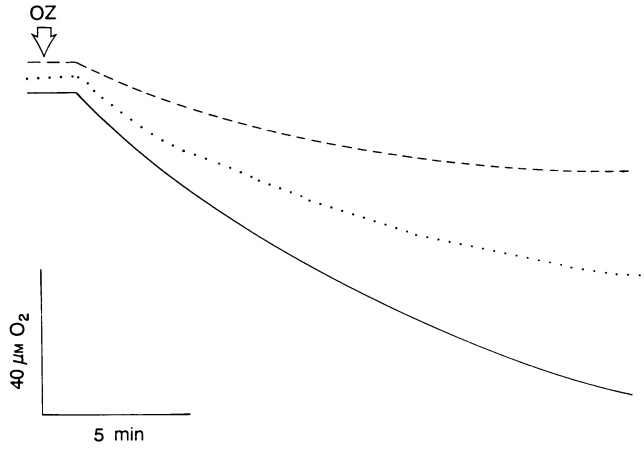

Figure 3. Oxygen consumption by OZ-activated neutrophils co-incubated without (——) and with 50 μm (· · · ·) and 100 μm (– – –) p201-206.

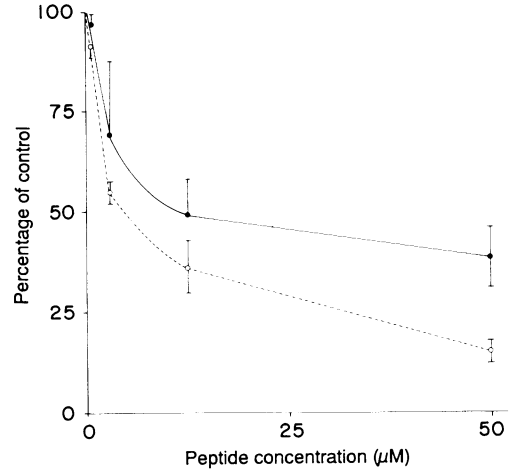

Figure 4. Effects of 0–50 μM of p77-82 (●) and p201-206 (○) on the activity of purified enolase. Data are from three separate experiments. A concentration of 1 mM 2PGA was used as substrate. In the absence of peptide 35·8±0·7 μM NAD^+ was produced/min.

Oxygen consumption

Both peptides inhibited oxygen consumption by OZ-activated neutrophils and data for p201-206 (50 and 100 μM, a single experiment) are shown in Fig. 3. The inhibition at 20 min was 32 and 63% of the peptide-free control for 50 and 100 μM of p201-206 respectively while the corresponding percentage inhibition for 50 and 100 μM of p77-82 was 28 and 63% respectively.

Intracellular lactate, ATP and activity of the glycolytic enzymes

These data shown in Fig. 1 clearly indicate that pretreatment of neutrophils with either p77-82 or p201-206 at a fixed concentration of 50 μM causes significant inhibition (approximately 30% for each peptide in each assay) of lactate ($P<0.005$) and ATP ($P<0.005$) production, as well as activity of the glycolytic enzyme enolase ($P<0.005$) in cytoplasmic extracts. The abso-

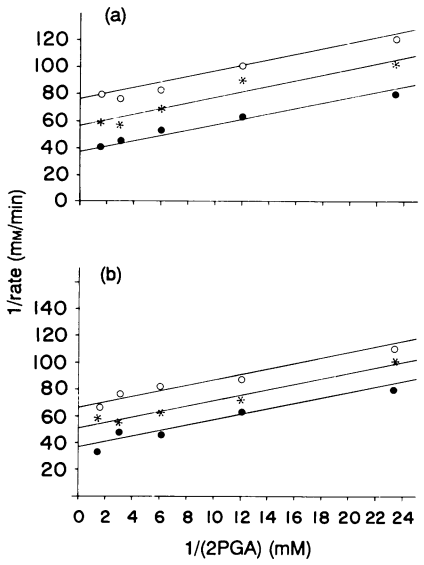

Figure 5. Lineweaver–Burk reciprocal plots of kinetics of purified enolase inhibition by (a) p201-206 and (b) p77-82 with 2PGA as substrate varying in concentration from 1 to 0·04 mM. 20 μM (○); 10 μM (*); 0 (●) of each peptide.

lute values of the respective peptide-free control systems and numbers of experiments for each test and the SEM are given in the legend to Fig. 1. The other glycolytic enzymes in neutrophil cytosolic extracts (see Materials and Methods) were unaffected when a concentration of 50 μM of p77-82 or p201-206 was used. However, when the concentration of p77-82 and p204-206 was increased to 100 μM, significant inhibition of cytosolic hexokinase ($27\pm6\%$ and $25\pm3\%$ respectively, $P<0{\cdot}005$, data from four experiments) and phosphofructokinase ($47\pm7\%$ and $40\pm10\%$ respectively, $P<0{\cdot}005$, data from four experiments) were observed. Increasing the concentration of p77-82 and p201-206 to 100 μM, resulted in the mean inhibition of neutrophil lactate production by each peptide, increasing respectively to $67\pm4\%$ and $77\pm12\%$ of the control value (data from two experiments, $P<0{\cdot}005$) (Fig. 1). Importantly neither of the control peptides, p70-74 and p160-165, each at concentrations of either 50 or 100 μM inhibited neutrophil lactate and ATP production or enolase activity. The respective values for control neutrophils of 45 ± 14 μg lactate/10^7 neutrophils, $2{\cdot}4\pm0{\cdot}4$ nmol ATP/10^7 cells and $3{\cdot}7\pm0{\cdot}4$ μM NAD^+/min/10^7 cells varied by less than 1% following pretreatment of the cells with 50 or 100 μM of either p70-74 or p160-165. In addition no inhibition of any of the other glycolytic enzymes, including hexokinase and phosphofructokinase was achieved when neutrophils were pretreated with p70-74 or p201-206 at these concentrations.

Pretreating neutrophils with 10 μg of a mixture of crude CRP peptides (produced by degrading CRP with a neutrophil membrane enzyme) known to contain p77-82 and p201-206[2] resulted in a reduction of ATP production in cytoplasmic extracts of these cells by $35\pm2\%$ (data from three experiments, $P<0{\cdot}005$).

The inhibitory effects of p77-82 and p201-206 (0–50 μM) on the activity of purified enolase, using 1 mM of 2-phosphoglyceric acid (2PGA) as substrate, is shown in Fig. 4. Inhibition was evident at a concentration of 0·7 μM and achieved statistical significance at 3 μM ($P<0{\cdot}01$) for both peptides. For p77-82 and p201-206, 50% inhibition was achieved at $12{\cdot}5\pm1$ μM and $4{\cdot}4\pm1$ μM respectively. The control peptides p70-74 and p160-165 did not exhibit this inhibitory activity on purified enolase. In a single experiment the nature of the inhibition of purified enolase activity by p77-82 and p201-206 was further investigated. Lineweaver–Burk reciprocal plots for 2PGA as substrate (1–0·041 mM) indicate that each peptide modifies both the V_{max} and K_m of the enzyme (Fig. 5). The pattern of inhibition for each peptide (a parallel upward shift of the kinetic plots, $r>0{\cdot}96$ for each respective kinetic plot) indicates that the inhibition is uncompetitive. A K_i of 19 ± 1 μM (Fig. 5a) and a K_i of $27\pm0{\cdot}1$ μM was calculated for p201-206 and for p77-82 (Fig. 5b) respectively.

Glucose uptake, arachidonate mobilization and transmembrane potential

The uptake of glucose by control neutrophils was $0{\cdot}36\pm0{\cdot}09$ nmol/2×10^6 cells per 30 min (three experiments) while the release of [^{3}H]arachidonate from unstimulated control neutrophils and cells exposed to 10 μg/ml clofazimine or 2·5 μM calcium ionophore was 93 ± 7, 213 ± 20 and 275 ± 5 fmol [^{3}H]arachidonate/5×10^7 neutrophils (three experiments) respectively. None of the peptides at the fixed concentration tested (50 μM) interfered with glucose uptake or release of [^{3}H]arachidonate in these neutrophils. Addition of PMA (10 ng/ml) to neutrophils to measure transmembrane potential was followed by a short lag phase (about 1 min) and then a typical abrupt decrease in fluorescence intensity of di-*O*-C_5(3) which was linear for about 2 min.[7] Co-incubation of neutrophils with 50 μM each of p77-82 or p201-206 or the control peptides did not affect either the rate or intensity of the PMA-induced alteration in neutrophil transmembrane potential (not shown). Increasing the concentration of these peptides to 100 μM did not alter any of these results.

DISCUSSION

We have previously reported that the synthetic peptides Val-Gly-Gly-Ser-Glu-Ile and Lys-Pro-Gln-Leu-Trp-Pro, which correspond to amino acid sequences at positions 77-82 and 201-206 respectively of the native CRP molecule, are generated as the result of the proteolytic cleavage of the acute phase reactant by a neutrophil membrane-associated protease.[1,2] Importantly these peptides have previously been shown to be potent stimulus non-specific inhibitors of neutrophil migration and superoxide generation.[1] Data from the present study show membrane-stabilizing effects are not involved in the biochemical mechanism of peptide-mediated inhibition of superoxide generation by activated neutrophils as the peptides did not interfere with glucose transport mechanisms or release of arachidonic acid from membrane phospholipids.[9] In addition, the peptides failed to alter neutrophil transmembrane potential accompanying activation of superoxide generation from FMLP/PMA-activated neutrophils.[10] Likewise, modulation of the initial transductional events, involved in activation of the oxidase, do not appear to be inhibited by these peptides since the initial kinetics of superoxide generation by OZ-activated neutrophils was seemingly unaffected by these agents. The observation that addition of the peptides to neutrophils, 5 min after activation with OZ, was accompanied by inhibition of superoxide generation supports this point.

From the experiments where the cells were preincubated with the peptides then washed prior to stimulation, it appears that the peptide-mediated inhibition of superoxide generation is time-dependent, a maximum being reached in 60 min. The ability of both p77-82 and p201-206 to inhibit oxygen consumption suggests the inhibition of superoxide generation by stimulated neutrophils is due to inhibition of NADPH-oxidase by the peptides. We have previously shown that these two peptides do not possess superoxide scavenging activity.[1]

The present results infer that interference with membrane-associated oxidative metabolism is related to the capacity of both peptides to inhibit neutrophil glycolysis and ATP generation. ATP involvement in maintaining superoxide generation is most probably related to a requirement for continuous phosphorylation of two cytosolic polypeptide co-factors necessary for NADPH-oxidase activity[11] while sustained generation of ATP is essential for neutrophil locomotion.[12] This observed depletion of ATP would imply that all ATP-dependent neutrophil functions are inhibited on pretreatment of the cells with these CRP peptides. The previously reported observation that these peptides inhibit Ca^{2+} mobilization in activated neutrophils[1] and the finding that CRP on incubation with stimulated neutrophils results in inhibition of phosphorylation of several cytosolic proteins[13] can therefore be explained, on the basis of the results from this study, to be events secondary to the initial CRP peptide-dependent depletion of ATP.

The observed inhibition of neutrophil glycolysis by these peptides appears to be the result of uncompetitive inhibition of the glycolytic enzyme enolase by both peptides rather than through modulation of active transport of glucose into neutrophils. The K_i for p77-82 of 27 μM being larger than the K_i of 19 μM for p201-206 is indicative that p77-82 is a weaker inhibitor of enolase than p201-206. This observed inhibition of neutrophil glycolysis however is specific for these amino acid sequences from CRP as other CRP peptides (p70-74 and p160-165) originating through neutrophil proteolysis of the parent molecule[1,2] failed to exhibit inhibition of this neutrophil biochemical process. The reasons for p77-82 and p201-206, which are very dissimilar amino acid sequences, possessing this activity are unclear. However peptides Lys-Pro-Arg and Gly-Ser-Glu, homologous to sequences within p201-206 and p77-82, have previously been shown to inhibit stimulation of human monocytes[14,15] and eosinophil and neutrophil chemotaxis[16] respectively.

Although the route of entry of the peptides into the neutrophil cytoplasm has not been elucidated the observed interference with enolase activity and subsequently ATP generation and glycolysis within the cytoplasm of intact neutrophils would suggest that an initial peptide membrane interaction is a prerequisite prior to entry of the peptides to the cytosol. Considering the hydrophobic character of the peptides,[1] it is conceivable that they can be taken up by neutrophils.

The concentration of 50 μM of each peptide required to inhibit neutrophil energy metabolism is possibly not easily attainable *in vivo*. During the acute phase response the serum concentration of CRP can rise to more than 250 μg/ml, i.e. 10 μM (subunit molecular weight 25,000). The ability of CRP to accumulate at sites of tissue damage[17] would suggest the CRP concentration at an inflammatory lesion is greater than that of circulating CRP levels. Our data clearly demonstrate that the inhibitory effects of p77-82 and p201-206 on superoxide generation by activated neutrophils increases with an extension of the preincubation period of the peptides with neutrophils. Consequently significant inhibition was achieved with as little as 6·25 μM of each peptide, concentrations attainable in vivo. In addition we have previously shown[1] that p77-82 and p201-206 act additively to inhibit neutrophil oxidative metabolism. Therefore it can be expected that since mixtures of these peptides will be formed *in vivo*, significant inhibition of neutrophil energy metabolism can be achieved at concentrations below 50 μM. The ability of a mixture of crude CRP peptides ($M_r < 14{,}000$, prepared by digesting CRP with PMA-activated neutrophils)[1] at a concentration of 10 μg/ml to effectively inhibit ATP generation supports this supposition. This mixture has been shown to contain amongst other peptides, peptides with amino acid sequences identical to the sequences of the bioactive synthetic peptides p77-82 and p201-206.[2]

The possibility that these bioactive CRP-derived peptides modulate other functions of activated neutrophils, such as neutrophil antimicrobial activity, is being investigated. However, the data presented here suggest that inhibition of neutrophil energy metabolism by CRP peptides and consequently inhibition of several functions of activated neutrophils, may be realized *in vivo* and beneficial in limiting the inflammatory response.

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