Effect of adrenaline and phorbol myristate acetate or bacterial lipopolysaccharide on stimulation of pathways of macrophage glucose, glutamine and O₂ metabolism

Evidence for cyclic AMP-dependent protein kinase-mediated inhibition of glucose-6-phosphate dehydrogenase and activation of NADP⁺-dependent 'malic' enzyme

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Adrenaline has recently been shown to stimulate both glucose metabolism and H₂O₂ release by macrophages but the activity of the key pentose phosphate pathway enzyme, glucose-6-phosphate dehydrogenase (which generates the NADPH crucial for the reduction of molecular oxygen), was reduced under these conditions [Costa Rosa, Safi, Cury and Curi (1992) *Biochem. Pharmacol.* **44**, 2235–2241]. We report here that adrenaline activates another NADPH-producing enzyme, NADP⁺-dependent 'malic' enzyme, while also inhibiting glucose-6-phosphate dehydrogenase, via cyclic AMP-dependent protein kinase (PKA) activation. Regulation of glucose-6-phosphate dehydrogenase activity by PKA has not been reported elsewhere. The sparing of

some glucose from pentose phosphate pathway consumption may be important in the provision of glycerol 3-phosphate which in the macrophage may be required for new phospholipid synthesis. Glutamine oxidation was also stimulated by adrenaline thus providing increased substrate (malate) for NADP⁺-dependent 'malic' enzyme and therefore shifting some of the burden of NADPH production from glucose to glutamine metabolism. We also report a novel synergistic effect of adrenaline and some bacterial products and/or γ -interferon in stimulating secretory and metabolic pathways in macrophages which may be a part of a larger network of signals that lead to enhanced macrophage activity.

INTRODUCTION

The role of cytokine networks in stimulating macrophage activity is widely recognized [1]. However, the interaction between hormonal and cytokine stimulation has been largely ignored. Indeed there have been few publications on macrophage activation by hormones alone. For example, the observation that adrenaline stimulated H₂O₂ release is reported in only one publication in recent years [2], while there are reports that adrenaline inhibited bacterial lipopolysaccharide (LPS) stimulation of tumour necrosis factor- α (TNF) release via β -adrenergic receptors [3] and down-regulated the TNF receptor number in macrophages [4] as well as activating a Ca²⁺-dependent K⁺ conductance via α 1-adrenoceptors [5]. As adrenaline may exert its effects through α - or β -adrenergic receptors and thus influence intracellular Ca²⁺ or cyclic AMP (cAMP) concentrations then it has been suggested that interaction between cytokine second messenger systems and adrenaline second messengers was possible, but little has been previously reported on this subject. The secretory profile of activated macrophages is impressive, resulting in the release of a large number of newly synthesized products including proteolytic enzymes, blood clotting factors, proteins of the complement pathway, cytokines, prostaglandins, thromboxanes and reactive metabolites of oxygen or nitrogen [6]. The secretion of O₂⁻ and its dismutation to H₂O₂ has been widely documented as a powerful anti-microbial and anti-tumour weapon [7,8]. The assay of released H₂O₂ is a convenient method for assessing the enhancement of macrophage anti-microbial activity by cytokines or other agents and is used in the work reported in this paper.

The source of reducing power for the reduction of molecular oxygen by a well-described phagocytic cell membrane-associated oxidase (see [9] for review) is NADPH. The NADPH is produced via the pentose phosphate pathway thus consuming glucose [10]. However, data from Costa Rosa et al. [2] has indicated that under conditions of adrenaline stimulation glucose-6-phosphate dehydrogenase activity is reduced to about 25% of control activity in macrophages elicited by BCG, while H₂O₂ release increased 2.6-fold. In the present paper we have measured the activity of another major NADPH-producing enzyme, NADP⁺-dependent malate dehydrogenase ('malic' enzyme), in adrenaline-activated macrophages as a possible alternative source of reducing power. Costa Rosa et al. [2] have also shown that adrenaline can increase macrophage glucose utilization by up to 6-fold while lactate production rates were not significantly different from control values. Thus glucose may be required for processes other than anaerobic glycolysis in conditions of adrenaline stimulation, including oxidation, amino acid synthesis and lipid synthesis. However, a large proportion of glutamine can be fully oxidized in the macrophage while only a relatively small proportion of glucose is utilized for oxidation [11,12]. Membrane turnover is increased dramatically in activated macrophages [13] thus increasing the demand for fatty acids for membrane lipid synthesis. We investigated the rate of macrophage glycerol release which was proportional to the rate of triacylglycerol hydrolysis as macrophages do not contain glycerol kinase (L. F. B. P. Costa Rosa, unpublished work). Hence the resynthesis of triacylglycerol will require glucose (via the provision of glycerol 3-phosphate). We have also measured glucose and glutamine oxidation to

Abbreviations used: BCG, Bacillus Calmette-Guérin; PMA, phorbol 12-myristate 13-acetate; TNF, tumour necrosis factor- α ; LPS, bacterial lipopolysaccharide; cAMP, 3',5'-cyclic adenosine monophosphate; O₂⁻, superoxide anion; FMLP, formyl-methionyl-leucyl-phenylalanine; db-cAMP, dibutyl cAMP; γ -IFN, γ -interferon; PGE₂, prostaglandin E₂; PKA, cAMP-dependent protein kinase; PKC, protein kinase C; SOD, superoxide dismutase.

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determine whether the rates of oxidation of these fuels were increased or decreased in conditions of adrenaline and cytokine stimulation.

MATERIALS AND METHODS

Chemicals and enzymes

All chemicals and enzymes were obtained from Sigma Chemical Company (U.S.A. or U.K.). [$U\text{-}^{14}\text{C}$]Glucose and [$U\text{-}^{14}\text{C}$]glutamine were purchased from Amersham International, U.K.

Animals

Male Wistar rats weighing 180 g (about 2 months of age) were obtained from the Biomedical Institute (USP, Sao Paulo, Brazil). The rats were maintained at 23 °C under a cycle of 12 h light: 12 h darkness.

Peritoneal cell preparation

Cells present in the intraperitoneal cavity of the rats were collected using 6 ml of phosphate-buffered saline (PBS). Activated cells were obtained by the lavage of the cavity 7 days after the administration of 50 mg of BCG (Bacille Calmette-Guérin; 500 mg/5 ml) obtained from the Butantan Institute, Sao Paulo, Brazil. Cell viability was confirmed by Trypan Blue exclusion (> 95%). At least 92% of the peritoneal exudate cells were macrophages as determined by differential counts.

Incubation procedure

Macrophages were incubated (1.0×10^8 cells/flask) at 37 °C in Krebs-Ringer medium with 2% defatted BSA in the presence of glucose (5 mM) or glutamine (2 mM). After incubation, the cells were disrupted by 0.2 ml of 25% perchloric acid. Protein was removed by centrifugation and the supernatant was neutralized with 2 ml of 40% KOH solution and a tris-(hydroxymethyl)aminomethane/KOH (0.5 to 2.0 M) solution for measurements of metabolites.

Metabolite measurements

Neutralized samples of the incubation medium were used for measurements of lactate [14] and glucose [15]. The method for glucose determination is based on the conversion into glucose 6-phosphate catalysed by hexokinase and the subsequent formation of 6-phosphogluconate and NADPH from glucose 6-phosphate and NADP^+ catalysed by glucose-6-phosphate dehydrogenase as reported by Bergmeyer et al. [15]. The lactate in the neutralized samples was measured using the method of Engel and Jones [14]. The production of NADH or NADPH was monitored at 340 nm, using a recording spectrophotometer (Gilford Response). By comparing the values at time zero and those obtained after 1 h incubation it was possible to calculate glucose consumption and lactate production by incubated macrophages. Glucose utilization or lactate production was linear over 1 h in the presence or absence of the compounds described in Table 2.

Glucose and glutamine oxidation

The $^{14}\text{CO}_2$ produced from [$U\text{-}^{14}\text{C}$]glucose and [$U\text{-}^{14}\text{C}$]glutamine was collected as described previously [16]. Macrophages were incubated for 1 h in the presence of one of the radiolabelled substrates in a sheltered Erlenmeyer (25 ml) presenting one compartment for cell incubation and another for CO_2 collection. After this period of time the cells were killed using 200 μl of

perchloric acid solution (25%). The labelled CO_2 was collected over 1 h in a solution of phenylethylamine methanol (1:1, v/v) and the radioactivity counted in a Beckman-LS 5000TD liquid scintillation counter (Beckman Instruments, Fullerton, CA, U.S.A.). The rate of production of CO_2 was linear over 1 h in all conditions described in Table 2 (L. F. B. P. Costa Rosa, unpublished work).

Glycerol production

The rate of glycerol production from macrophages was measured as described previously [17]. The assay medium consisted of 0.1 M Tris/HCl, 4 mM MgSO_4 , 0.3 mM NAD^+ , 100 mM phosphoenolpyruvate, 100 mM ATP; 5 units of pyruvate kinase and glycerol-3-phosphate dehydrogenase, pH 7.6. To half of the samples glycerol kinase (5 mg/ml) was added. The samples were then incubated for 1 h at room temperature, and glycerol production calculated from the difference in NADH production between the samples containing glycerol kinase and those without the enzyme. The rate of production of glycerol was linear over 1 h in all conditions described in Table 2 (L. F. B. P. Costa Rosa, unpublished work).

H_2O_2 release

The release of H_2O_2 was measured by a modification of the method described by Pick and Mizel [18] and is described elsewhere [2]. Briefly, the cells were incubated in siliconized flasks (25 ml), in 1 ml of PBS, in the presence of glucose (5 mM), under an atmosphere of 5% CO_2 /95% air at 37 °C and additionally, in some cases, in the presence of the various compounds described in Table 2. After 1 h incubation, a solution of Phenol Red and horseradish peroxidase (HRPO) was added to the medium to quantify the H_2O_2 content. After a further 10 min, the reaction was stopped with 100 μl of 1 M NaOH and the amount of H_2O_2 formed was measured spectrophotometrically at 620 nm. The rate of release of H_2O_2 was linear over a period of 1 h and there was no significant difference in H_2O_2 release if the cells were incubated in the presence or absence of bicarbonate-containing medium (L. F. B. P. Costa Rosa, unpublished work).

Enzyme assays

Enzyme activities were determined after a 1 h incubation in the conditions indicated in Table 1. The cells were homogenized in extraction medium using a hand-held homogenizer as described previously [2,12,15]. The assay medium for glutaminase (EC 3.5.1.2) consisted of 50 mM phosphate buffer (mixture of KH_2PO_4 and K_2HPO_4), 0.2 mM EDTA, 50 mM Tris/HCl, and 20 mM glutamine, pH 8.6. The final volume of the assay mixtures in all cases was 1.0 ml. The activity of the enzyme was evaluated through an increase in NADH produced in the reaction catalysed by glutamate dehydrogenase [19]. Glucose-6-phosphate dehydrogenase (EC 1.1.1.49) activity was measured as described by Bergmeyer et al. [15]. The assay medium consisted of 86 mM Tris/HCl, 6.9 mM MgCl_2 , 0.4 mM NADP^+ , 1.2 mM glucose 6-phosphate and 0.05% (v/v) Triton X-100 at pH 7.6. NADP^+ -dependent 'malic' enzyme (EC 1.1.1.82) activity was determined as described by Newsholme and Williams [20]. The assay medium consisted of 50 mM Tris/HCl, 1 mM MgCl_2 , 0.2 mM NADP^+ , 100 mM KCl, 0.05% (v/v) Triton X-100 and 2 mM malate (neutralized with KOH).

All spectrophotometric measurements were performed using a Gilford (Response) recording spectrophotometer at 25 °C, except for glutaminase activity which was determined at 37 °C. Preliminary experiments established that extraction and assay pro-

Table 1 The effect of PMA, db-cAMP, adrenaline, KCl, bacterial products and γ -interferon on the activities of some enzymes in the pathways of glucose or glutamine metabolism

Macrophages were elicited by BCG injected intraperitoneally 7 days prior to harvest. Values shown are mean $\pm$ S.E.M. of data obtained from 12 incubations. Enzyme activity measurements are described in the Materials and methods section, and were obtained after macrophages were incubated for 1 h in the presence of the above stimulators. N.D.; not determined.

Conditions	Enzyme activities (nmol/min per mg of protein)		
	Glucose-6-phosphate dehydrogenase	NADP ⁺ -dependent 'malic' enzyme	Glutaminase
No additions	18.80 $\pm$ 1.50	3.0 $\pm$ 0.2	194.1 $\pm$ 6.0
PMA (10 ng/ml)	34.70 $\pm$ 0.60	5.0 $\pm$ 0.17	235.4 $\pm$ 11.0
Adrenaline (5 nM)	4.9 $\pm$ 0.31	9.1 $\pm$ 0.62	244.3 $\pm$ 17.0
Adrenaline + PMA	10.0 $\pm$ 0.70	13.4 $\pm$ 0.7	254.9 $\pm$ 12.9
Db-cAMP (100 μ M)	4.0 $\pm$ 0.13	7.0 $\pm$ 0.21	N.D.
Db-cAMP + PMA	9.5 $\pm$ 0.41	11.1 $\pm$ 0.74	257.8 $\pm$ 16.9
KCl (30 mM)	22.4 $\pm$ 2.30	5.1 $\pm$ 0.32	N.D.
FMLP (100 μ g/ml)	17.3 $\pm$ 1.54	2.9 $\pm$ 0.16	189.7 $\pm$ 7.4
FMLP + adrenaline	9.5 $\pm$ 0.65	9.2 $\pm$ 0.21	236.1 $\pm$ 15.2
γ -IFN (1000 U/ml)	16.1 $\pm$ 1.02	2.3 $\pm$ 0.20	194.2 $\pm$ 3.9
γ -IFN + adrenaline	5.1 $\pm$ 0.19	8.4 $\pm$ 0.33	238.7 $\pm$ 13.9
LPS (15 μ g/ml)	27.5 $\pm$ 1.04	2.1 $\pm$ 0.24	229.7 $\pm$ 12.3
LPS + adrenaline	12.3 $\pm$ 0.65	9.0 $\pm$ 0.32	237.2 $\pm$ 14.0
LPS + γ -IFN	29.7 $\pm$ 1.34	3.0 $\pm$ 0.27	226.7 $\pm$ 11.5
LPS + γ -IFN + adrenaline	20.1 $\pm$ 1.45	8.7 $\pm$ 0.43	245.1 $\pm$ 16.4

cedures were so as to produce maximum enzyme activities for all enzymes studied [21].

Expression of results

The enzyme activities are expressed as nmol of substrate utilized/min per mg of protein. Protein concentration was determined using a Sigma protein assay kit (Cat. No. P5656). The rates of glucose consumption, lactate, glycerol and H₂O₂ formation and of oxidation of [U-¹⁴C]glucose and [U-¹⁴C]glutamine are presented as nmol/h per 10⁶ cells. Analysis of the significance of differences with respect to control experiments, using a standard Student's *t*-test, are given in the text.

RESULTS

Enzyme activity changes

Addition of the phorbol ester phorbol 12-myristate 13-acetate (PMA) (10 ng/ml) to BCG-activated macrophages caused a 2-fold increase in activity of macrophage glucose-6-phosphate dehydrogenase activity and a 1.6-fold increase in 'malic' enzyme activity (Table 1). The addition of 5 nM adrenaline to BCG-activated macrophages resulted in a 4-fold decrease in glucose-6-phosphate dehydrogenase activity ($P < 0.01$ with respect to no adrenaline control) while 'malic' enzyme activity was increased 3-fold ($P < 0.01$ with respect to the no adrenaline control; Table 1). As these effects on maximal enzyme activity may have been mediated via cAMP or Ca²⁺ second messenger pathways then experiments were performed with the cAMP analogue dibutyryl cAMP (db-cAMP) or 30 mM extracellular KCl (a concentration that will shift the resting membrane potential of the cell to approximately -30 mV, thus opening voltage-dependent Ca²⁺ channels). The results of these experiments are shown in Table 1 and demonstrate that db-cAMP can mimic the effects of adrenaline on the activities of glucose-6-phosphate dehydrogenase but KCl can not. There is a synergistic effect of adrenaline and PMA, or db-cAMP and PMA, on the activity of 'malic' enzyme, but

PMA antagonized the inhibitory effect of adrenaline or db-cAMP on the activity of glucose-6-phosphate dehydrogenase (Table 1).

The bacterial products formyl-methionyl-leucyl-phenylalanine (FMLP) or LPS were assessed for their ability to affect NADPH-producing enzyme activity. LPS, which increases the secretion of inflammatory products from macrophages, increased the activity of glucose-6-phosphate dehydrogenase by 1.5-fold ($P < 0.01$). FMLP, which is a chemotactic attractant for macrophages, had no effect on enzyme activity. However, both bacterial products antagonized the inhibitory effect of adrenaline on glucose-6-phosphate dehydrogenase activity while they had no effect on the adrenaline-stimulated increase in 'malic' enzyme activity. γ -Interferon (γ -IFN) has little effect on macrophage enzyme activity when added alone and appears not to antagonize the effect of adrenaline or LPS (Table 1). The activity of glutaminase was also measured as it is known that glutamine metabolism plays an important part in macrophage energy and nitrogen provision [11,22–24]. The activity of glutaminase was increased 10–25% by most stimulatory molecules except FMLP and γ -IFN ($P < 0.05$, Table 1).

Changes in fuel utilization rates and production rates of secretory products

Rates of glucose utilization and H₂O₂ release as well as lactate production were elevated by the addition of 5 nM adrenaline ($P < 0.01$ for all conditions, Table 2). Adrenaline also stimulates the rate of glucose oxidation, glycerol production and glutamine oxidation ($P < 0.01$ for all conditions, Table 2). The rates of utilization of glucose and release of lactate, glycerol or H₂O₂ were linear over a period of 60 min (see Figure 1). There was a synergistic effect of adrenaline and PMA on H₂O₂ production (Table 2) while the effect of PMA on adrenaline-enhanced glycerol production was antagonistic. The stimulatory effect of adrenaline on macrophage glucose or glutamine oxidation, or H₂O₂ release, could be imitated by cAMP but not by KCl ($P < 0.01$, Table 2). FMLP or γ -IFN had no effect on macrophage glucose metabolism but γ -INF stimulated glutamine oxidation 1.5-fold ($P < 0.01$), whereas FMLP had no effect on glutamine oxidation. Adrenaline or db-cAMP also stimulated glutamine oxidation by approximately 2-fold ($P < 0.01$). FMLP or γ -IFN do not appear to antagonize or enhance the effects of adrenaline on glucose or glutamine metabolism with the exception of an antagonism of adrenaline-enhanced glycerol production. LPS enhanced rates of glucose utilization, plus lactate and H₂O₂ release ($P < 0.01$). LPS and adrenaline appeared to act synergistically to enhance rates of lactate, glycerol and H₂O₂ release and also synergistically enhance glutamine oxidation (Table 2). The synergistic effect of γ -IFN on LPS-stimulated changes in glucose utilization and metabolism and glutamine oxidation is also shown in Table 2, but γ -IFN was not as effective as adrenaline at enhancing the rate of production of glycerol or oxidation of glutamine; however, it was as effective in stimulating H₂O₂ production.

Effect of cAMP-dependent phosphorylation on the activities of macrophage glucose-6-phosphate dehydrogenase and NADP⁺-dependent 'malic' enzyme

The decrease in activity of glucose-6-phosphate dehydrogenase and increase in activity of NADP⁺-dependent 'malic' enzyme stimulated by adrenaline or cAMP, and reported in Table 1, may have occurred via PKA-dependent phosphorylation. To investigate this possibility macrophages were incubated with db-cAMP, or digitonin-permeabilized macrophages were incubated

Table 2 The effect of PMA, db-cAMP, adrenaline, KCl, bacterial products or γ -interferon on the rates of glucose utilization and oxidation, lactate, glycerol and H_2O_2 production and glutamine oxidation

All rates are expressed as nmol/h per 10^6 cells. Values shown are means $\pm$ S.E.M. of data obtained from 12 incubations. Metabolite measurements are described in the Materials and methods section and were obtained after 1 h incubation in the presence of the above stimulators.

Conditions	Glucose utilization	Glucose oxidation	Lactate production	Glycerol production	H_2O_2 release	Glutamine oxidation
No additions	273 $\pm$ 18.4	47.2 $\pm$ 3.0	280 $\pm$ 15.3	60 $\pm$ 3.4	12.1 $\pm$ 0.70	17.3 $\pm$ 0.9
PMA (10 ng/ml)	387 $\pm$ 21.9	51.2 $\pm$ 2.8	351 $\pm$ 20.1	58 $\pm$ 4.2	38.2 $\pm$ 2.10	18.2 $\pm$ 1.1
Adrenaline (5 nM)	981 $\pm$ 46.3	108.3 $\pm$ 9.2	342 $\pm$ 6.7	142 $\pm$ 8.7	18.4 $\pm$ 1.03	31.3 $\pm$ 2.7
Adrenaline + PMA	974 $\pm$ 43.2	124.5 $\pm$ 8.7	338 $\pm$ 22.6	62 $\pm$ 5.6	52.4 $\pm$ 0.90	34.6 $\pm$ 2.1
db-cAMP (100 μM)	418 $\pm$ 5.1	117.2 $\pm$ 9.8	165 $\pm$ 7.2	N.D.	23.1 $\pm$ 1.90	40.1 $\pm$ 2.5
KCl (30 mM)	253 $\pm$ 3.2	51.2 $\pm$ 1.9	257 $\pm$ 3.6	N.D.	11.6 $\pm$ 1.01	17.2 $\pm$ 0.9
FMLP (100 $\mu\text{g}/\text{ml}$)	279 $\pm$ 15.7	46.2 $\pm$ 2.1	287 $\pm$ 17.8	50 $\pm$ 3.2	13.8 $\pm$ 1.02	19.4 $\pm$ 1.5
FMLP + adrenaline	945 $\pm$ 32.1	105.6 $\pm$ 4.2	387 $\pm$ 21.9	49 $\pm$ 3.1	17.2 $\pm$ 1.40	30.2 $\pm$ 1.9
γ -IFN (1000 U/ml)	265 $\pm$ 16.3	45.3 $\pm$ 2.6	274 $\pm$ 16.3	51 $\pm$ 6.5	11.0 $\pm$ 0.54	26.7 $\pm$ 1.1
γ -IFN + adrenaline	895 $\pm$ 34.6	110.2 $\pm$ 9.3	398 $\pm$ 18.6	50 $\pm$ 3.3	18.6 $\pm$ 0.54	30.2 $\pm$ 1.4
LPS (15 $\mu\text{g}/\text{ml}$)	406 $\pm$ 23.8	57.8 $\pm$ 1.8	332 $\pm$ 17.3	52 $\pm$ 3.7	18.4 $\pm$ 1.20	18.4 $\pm$ 1.2
LPS + adrenaline	879 $\pm$ 67.4	145.6 $\pm$ 8.7	401 $\pm$ 21.8	129 $\pm$ 6.5	27.9 $\pm$ 0.76	45.6 $\pm$ 2.1
LPS + γ -IFN	381 $\pm$ 23.1	78.9 $\pm$ 3.2	391 $\pm$ 18.5	95 $\pm$ 7.6	31.2 $\pm$ 1.40	29.8 $\pm$ 1.5
LPS + γ -IFN + adrenaline	901 $\pm$ 34.8	170.0 $\pm$ 5.4	299 $\pm$ 16.4	151 $\pm$ 8.2	48.3 $\pm$ 1.90	50.1 $\pm$ 2.6

Figure 1 Time course of metabolite utilization or release in response to 5 nM adrenaline

Cells were incubated in the presence of 5 mM glucose for a period of 60 min, with adrenaline. Glucose utilization (●), lactate production (△), glycerol production (○) and H_2O_2 release (□) were measured 15, 30 and 60 min after the start of the incubation as described in the Materials and methods section.

in the presence of the catalytic subunit of PKA, or macrophage homogenates were incubated with the catalytic subunit of PKA with or without digitonin (Table 3). The inhibitory effect of PKA on glucose-6-phosphate dehydrogenase activity is dramatic, the activity of the enzyme being reduced to approximately 12% of control activity ($P < 0.01$, Table 3). The stimulatory effect of PKA on macrophage NADP^+ -dependent 'malic' enzyme activity was also observed (Table 3), increasing activity approximately 3-fold over control values ($P < 0.01$). As there are no previous reports of PKA-induced activity changes in mammalian glucose-6-phosphate dehydrogenase or NADP^+ -dependent 'malic' enzyme we assessed the ability of PKA to cause changes in activity of these enzymes from control tissues rich in these enzymes, bovine adrenal glands and chicken liver respectively. There was

Table 3 Effect of PKA-induced phosphorylation on the activities of macrophage glucose-6-phosphate dehydrogenase and 'malic' enzyme

Macrophages were elicited by BCG injected intraperitoneally 7 days prior to harvesting. All rates are expressed as nmol/min per mg of protein. Values shown are means of data obtained from 12 separate measurements. Enzyme activity measurements are described in the Materials and methods section. Whole cells, digitonin (0.5 μM)-permeabilized macrophages or macrophage homogenates were incubated for 15 min at 25 °C in the presence or absence of the indicated concentrations of PKA catalytic subunit, 25 mM Tris/HCl, pH 7.4, 1.3 mM dithiothreitol and 5 mM Mg/ATP before determination of activity. Cells that had not been homogenized at the start of the incubation period were homogenized after the 15 min incubation.

Conditions	Enzyme activity	
	Glucose-6-phosphate dehydrogenase	NADP^+ -dependent 'malic' enzyme
Whole cell or cell homogenate + no additions	19.70 $\pm$ 1.30	3.78 $\pm$ 0.31
Whole cell + adrenaline (5 nM)	4.27 $\pm$ 0.35	9.32 $\pm$ 0.65
Whole cell + db-cAMP (100 μM)	4.01 $\pm$ 0.13	7.03 $\pm$ 0.21
Cell homogenate + PKA catalytic subunit (2.5 U/ml)	2.45 $\pm$ 0.12	10.70 $\pm$ 0.84
Digitonin-permeabilized whole cell	18.5 $\pm$ 1.30	3.66 $\pm$ 0.23
Digitonin-permeabilized whole cell + PKA catalytic subunit	2.38 $\pm$ 0.17	11.80 $\pm$ 0.76

no change in activity compared with control values at a low (5 units) concentration of PKA catalytic subunit (Table 4); thus the macrophage isoenzymes of these two NADPH -producing enzymes appear to be unique (cf. Table 3, +PKA).

DISCUSSION

Phorbol ester stimulation of the macrophage respiratory burst is a well-defined phenomenon (see [9]). There is a tight coupling between NADPH production via the pentose phosphate pathway and the activity of the membrane-bound NADPH oxidase which generates the O_2^- critical for anti-tumour and anti-microbial activity. Thus the finding that PMA (10 ng/ml) caused a 2-fold increase in activity of macrophage glucose-6-phosphate dehydrogenase activity was expected (Table 1). The increase in activity of

Table 4 Effect of PKA-induced phosphorylation on the activities of non-macrophage isoenzymes of glucose-6-phosphate dehydrogenase and 'malic' enzyme

Activities are expressed as U (nmol/min) per ml except where otherwise defined. Values shown are means of data obtained from at least three separate measurements. Enzyme activity measurements are described in the Materials and methods section. Bovine adrenal gland glucose-6-phosphate dehydrogenase and chicken liver NADP⁺-linked 'malic' enzyme were purchased from Sigma chemical company and were incubated for 15 min at 25 °C in the presence of the indicated concentrations of PKA catalytic subunit, 25 mM Tris/HCl, pH 7.4, 1.3 mM dithiothreitol and 5 mM Mg/ATP before determination of activity.

Concentration of catalytic subunit of PKA in assay	Enzyme activity	
	Glucose-6-phosphate dehydrogenase	NADP ⁺ -dependent 'malic' enzyme
0 U (pmol of phosphate/min)	205 ± 4.36	24.0 ± 0.16
5 U	201 ± 10.07	25.3 ± 0.17
10 U	198 ± 5.03	26.5 ± 0.23
20 U	189 ± 4.36	36.5 ± 0.61
50 U	182 ± 3.00	39.4 ± 1.49
100 U	201 ± 3.46	44.7 ± 1.02

the latter enzyme (measured in conditions of maximal substrate and allosteric activator concentrations) may reflect a change in the protein kinase C (PKC)-stimulated phosphorylation state, increasing the capacity of the pentose phosphate pathway to generate NADPH. The unexpected decrease in activity of glucose-6-phosphate dehydrogenase activity in BCG-elicited macrophages stimulated by adrenaline has previously been reported [2]. However, in the latter study adrenaline was used at the pharmacological concentration of 43 μ M, while in this work we report similar results using the physiological concentration of 5 nM adrenaline (Table 1). The changes in enzyme activity reported in Tables 1 and 3 indicate that some key enzymes involved in macrophage glucose and glutamine metabolism are altered by PKA-induced phosphorylation. The PKA-induced inhibition of glucose-6-phosphate dehydrogenase and activation of NADP⁺-dependent 'malic' enzyme may indicate a shift in NADPH production from the former enzyme to the latter. Glucose may be required for processes unconnected with the pentose phosphate pathway during adrenaline stimulation, thus NADPH will need to be generated by alternative pathways, for example glutamine may be used to provide malate for NADP⁺-dependent 'malic' enzyme, as part of the glutamine oxidation pathway. Macrophage glutaminase, which may play a regulatory role in the pathway of glutamine oxidation, is only inhibited by high glutamate concentrations (IC₅₀ of 20 mM) and is involved in generating a large 'store' of glutamate which is subsequently slowly oxidized [12]. It also displays increased activity in the same conditions as enhance 'malic' enzyme activity (Table 1), although the activity of glutaminase is increased by a smaller factor (but its unstimulated activity is much greater). It is generally considered that the pentose phosphate pathway may generate up to 12 molecules of NADPH per glucose molecule oxidized (requiring six sequential passes through the pathway). However, as the product of each oxidation step, ribulose 5-phosphate, must be converted back into glucose 6-phosphate before re-entering the pentose phosphate pathway, then it is the activity of glucose-6-phosphate dehydrogenase that dictates the rate of generation of NADPH. The requirement of NADPH generation for H₂O₂ formation may be calculated from the rate of H₂O₂ release (assuming a stoichiometry of one molecule of NADPH required for each molecule of H₂O₂ formed) i.e. in

unstimulated conditions 12.1 nmol of H₂O₂ are released/h per 10⁶ cells thus requiring 12.1 nmol of NADPH. We may calculate the theoretical maximal NADPH production from the maximal activities of glucose-6-phosphate dehydrogenase and 'malic' enzyme (given that 1 mg of protein is equivalent to 10⁷ cells, [12]). There are two molecules of NADPH produced from the early stages of the pentose phosphate pathway leading to the formation of pentose phosphate, thus for every molecule of glucose 6-phosphate metabolized via this pathway two molecules of NADPH are produced. Thus, in unstimulated conditions, the pentose phosphate pathway may provide 226 molecules of NADPH/h per 10⁶ cells while 'malic' enzyme can provide 18 nmol/h per 10⁶ cells (i.e. the pentose phosphate pathway can provide 92 %, and NADP⁺-dependent 'malic' enzyme 8 %, of the theoretical maximum amount of NADPH). However, under conditions where macrophages have been stimulated by 5 nM adrenaline then the requirement of NADPH for H₂O₂ release increases to 18.4 nmol/h per 10⁶ cells. Under these conditions, due to a decrease in glucose-6-phosphate dehydrogenase activity and an increase in NADP⁺-dependent 'malic' enzyme activity then the pentose phosphate pathway will provide up to 59 nmol/h per 10⁶ cells whereas 'malic' enzyme will provide up to 55 nmol/h per 10⁶ cells (i.e. each pathway can provide about 50 % of the theoretical maximum amount of NADPH). The predicted excess capacity of NADPH production is probably related to a requirement for reduction of oxidized glutathione via glutathione reductase. The inhibition of macrophage glucose-6-phosphate dehydrogenase activity by PKA-induced phosphorylation that we have reported in this work is unique to the macrophage isoenzyme, as no such inhibition has been reported for any other isoenzyme. We have shown that under identical conditions PKA-induced inhibition of bovine adrenal gland isoenzyme does not occur (Table 4). cAMP acting via PKA-induced phosphorylation and activation of NADP⁺-dependent 'malic' enzyme is still not widely recognized as a mechanism of activation of the latter enzyme and we report here that the activity of chicken liver 'malic' enzyme increased slowly with PKA concentration, reaching a 2-fold increase in activity when incubated with 100 units of PKA catalytic subunit (Table 4). The macrophage isoenzyme of NADP⁺-dependent 'malic' enzyme, however, required incubation with a much lower (2.5 units) concentration of PKA catalytic subunit to achieve a 3-fold increase in activity (Table 3). The data presented in Tables 1 and 3 suggest the macrophage isoenzymes of these two NADPH-producing enzymes appear to be sensitive to PKA, activated via adrenaline binding to β -adrenergic receptors.

The effect of cAMP in macrophages, acting by stimulation of PKA, has previously been considered to be suppressive, for example prostaglandin E₂ (PGE₂) elevates cAMP and suppresses the anti-tumour function and the ability to present antigen [25,26]. PGE₂ can also antagonize the action of γ -IFN. However, no work has previously reported the effect of other cAMP-elevating agents on macrophage metabolism or function. In this paper we have studied metabolic and secretory events that occur rapidly (within 1 h) after exposure to a stimulus. We have shown that adrenaline acting via elevated cAMP levels was stimulatory with respect to enhanced glucose and glutamine oxidation and H₂O₂ release. Adrenaline also had a synergistic effect on H₂O₂ release when added with PMA or had a stimulatory effect when added with LPS, agents that act via stimulation of PKC (Table 2). Adrenaline also had a synergistic effect on rates of H₂O₂ release, glucose oxidation and glutamine oxidation when added with LPS and γ -IFN (Table 2). As superoxide dismutase (SOD) activity may be altered by adrenaline, thus altering the rate of release of H₂O₂ without altering the activity

of the macrophage NADPH oxidase, then we measured the activity of KCN-sensitive SOD (Cu-Zn-dependent isoenzyme) and KCN-insensitive SOD (Fe-Mn-dependent isoenzyme) in the presence of increasing concentrations of O_2^- . There was no significant effect of 5 nM adrenaline on the activity of the enzyme in the presence of any of the concentrations of O_2^- used (results not shown). Thus adrenaline must have altered the rate of molecular oxygen conversion to O_2^- by altering the activity of the macrophage NADPH oxidase. The presence of KCN did not alter the activity of the macrophage SOD (results not shown), suggesting that the macrophage isoenzyme of SOD is fully Fe-Mn dependent.

Macrophages can be stimulated by a large number of inflammatory agents including bacterial products and cytokines. The mechanism of signal transduction and subsequent activation is known for a small number of these agents including LPS and γ -IFN. One of the primary effects of γ -IFN is to increase the potential of PKC for activation [27], it also raises intracellular diacylglycerol concentration via stimulation of phosphatidylcholine breakdown as well as stimulating rates of gene transcription and translation which can lead to enhanced expression of MHC II proteins, antigen presentation and cytokine receptors on the cell surface [27,28]. LPS can lead to an increase in intracellular Ca^{2+} via polyphosphoinositide release from polyphosphatidylinositol and activation of PKC [29].

The stimulatory effect of phorbol esters on macrophage H_2O_2 release has been previously reported [9,30], as has the enhanced glucose utilization required for the increased activity of the pentose phosphate pathway. When BCG-activated macrophages were exposed to the phorbol ester PMA an enhancement of glucose utilization, lactate and H_2O_2 release was observed (Table 2). Adrenaline, however, stimulated an increase in all the measured parameters, while adrenaline and PMA acted synergistically to further enhance H_2O_2 production. Only the combination of adrenaline and LPS, or adrenaline, LPS and γ -IFN enhanced rates of glucose and glutamine oxidation above adrenaline alone, while the same two combinations stimulated the rate of glycerol production to a level observed with adrenaline alone. Glycerol production, as reported here, may have been proportional to the rate of triacylglycerol hydrolysis, as macrophages do not possess glycerol kinase (L. F. B. P. Costa Rosa, unpublished work). Adrenaline raises the rate of glycerol production, probably reflecting an increase in activity of triacylglycerol lipase. However, agents that promote PKC activation, e.g. PMA or γ -IFN but not LPS, lowered adrenaline-stimulated glycerol production back to control values (Table 2). The combination of adrenaline and LPS, or adrenaline, LPS and γ -IFN, however, stimulated glycerol production close to the level observed in the presence of adrenaline only and may reflect the different phosphorylation and activation patterns under these conditions. The hydrolysis of triacylglycerol leading to glycerol release will also result in increased fatty acid availability, which may be used for new phospholipid synthesis essential to the process of enhanced membrane turnover observed in activated macrophages [13]. The glycerol 3-phosphate required for new membrane synthesis (or triacylglycerol synthesis) may represent an alternative fate for utilized glucose, accounting for between 7% and 12% of consumed glucose. We have found that [U - ^{14}C]glucose incorporation into phospholipid is increased 2–3-fold in conditions of adrenaline stimulation (results not shown). Lactate production has been previously reported as a major fate for utilized glucose [11] accounting for approximately 90% of

glucose in thioglycollate-elicited macrophages. However, Costa Rosa et al. [2] have reported that BCG-elicited and adrenaline-activated macrophages metabolize only 15–17% of utilized glucose to lactate, an observation confirmed in the present study. Indeed in all conditions where adrenaline was added, 52–64% of utilized glucose could not be accounted for, compared with 20% in control (no additions) macrophages. The fate of the unaccounted glucose is presently under investigation in our laboratories.

The work described in this paper has identified some novel aspects of adrenaline activation of macrophage glucose, glutamine and oxygen metabolism. The interaction of PKA- and PKC-mediated events described here may have important implications for modulating macrophage activity in inflammatory conditions.

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