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Stimulation of glycolysis as an activation signal in rat peritoneal macrophages

Effect of glucocorticoids on this process

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1. Peritoneal macrophages were prepared from control, *Escherichia coli*-treated and triamcinolone acetonide-treated rats. Control and *E. coli*-treated rats produced resident and activated macrophages respectively. Glycolysis in these cells was studied by the fructose 2,6-bisphosphate (Fru-2,6-P_2) content, lactate release and 6-phosphofructo-1-kinase (PFK-1) and 6-phosphofructo-2-kinase (PFK-2) activities. 2. In activated macrophages, lactate release and Fru-2,6-P_2 content were increased several-fold compared with those in resident cells. Moreover, the response of these parameters to phorbol 12-myristate 13-acetate in activated macrophages was greater than for resident cells. 3. PFK-2 activity was moderately increased (about 3-fold), but PFK-1 activity was increased 5-fold in activated macrophages compared with resident cells. Partially purified preparations of PFK-1 were sensitive to Fru-2,6-P_2, with $K_{0.5}$ about 0.25 μM in both control and activated cells. However, the $V_{max.}$ of PFK-1 from activated cells was increased. In addition, AMP stimulated PFK-1, but the kinetic pattern was different from that described for Fru-2,6-P_2. Moreover there was no difference in the stimulation by AMP of PFK-1 from resident and activated cells. 4. Fru-2,6-P_2 content and lactate release in macrophages from triamcinolone acetonide-treated rats were decreased in both resident and activated cells. Also, the glucocorticoid inhibited PFK-1 and PFK-2 activities in both resident and activated macrophages. PFK-1 from triamcinolone acetonide-treated rats was not stimulated by Fru-2,6-P_2, whereas the effect of AMP was unchanged. The effects of glucocorticoid seem to be specific for phagocytic cells, since the glucocorticoid treatment increased PFK-1 and PFK-2 activities in liver.

INTRODUCTION

The term 'activated macrophages' was first defined by Mackaness, and studies have been undertaken to clarify its nature. Activated macrophages exhibit several specific characteristics compared with resting cells. These include changes in their morphology, a greater bactericidal power, an increased uptake of glucose and oxygen, and a stimulation of specific protein secretion such as proteinases and lysosomal enzymes. The modifications in these parameters seem to be a prerequisite to increase phagocytic capacity in the activated state of macrophages (for reviews see [1–4]).

Experimental models to produce activation in these cells relies on the bacterial infection of the peritoneum (e.g. with *Listeria*, *Bacillus Calmette-Guérin* or *Escherichia coli* [5]). It has been shown that the release of lactate as an index of glycolysis is increased in activated macrophages [6]. However, little is know about the molecular mechanisms which underlie this phenomenon. Previously we have shown that regulatory metabolites of the glycolytic pathway [e.g. fructose 2,6-bisphosphate (Fru-2,6-P_2)] are sensitive to glucose, phorbol esters and other agents in macrophages [7]. The present study was undertaken to characterize further the nature of the modification observed of the glycolytic pathway in activated macrophages. We show that in activated cells Fru-2,6-P_2 content and lactate release are increased, as are the activities of two key glycolytic enzymes, 6-phosphofructo-1-kinase (PFK-1) and 6-phosphofructo-2-kinase (PFK-2). We suggest that these parameters could be taken as an index of the activated state of macrophages, in addition to others which have been described [1–4].

It is known that glucocorticoids inhibit important functions in different tissues, including lymphoid cells [8,9]. In macrophages these glucocorticoids co-operate with other immunosuppressors (e.g. cyclosporine A) to decrease the respiratory burst [10]. In thymus lymphocytes, they inhibit glycolytic flux by decreasing both the numbers of available glucose carriers [11] and Fru-2,6-P_2 content [12]. We have studied the relationships between the effect of glucocorticoids and the activation of macrophages on some specific parameters of glycolysis. The present data demonstrate that triamcinolone acetonide, an artificial glucocorticoid, drastically inhibits glycolysis. In addition, it selectively modulates the action of the positive effectors, Fru-2,6-P_2 and AMP, on PFK-1 activity. In summary, the present data are in agreement with the observation that glucocorticoids specifically reverse those biochemical functions which had been increased in the activated state of macrophages.

MATERIALS AND METHODS

Animals and treatments

Male Wistar rats (170–200 g) were used throughout. Rats were either untreated (resident cells) or injected intraperitoneally with 2 ml (2×10^9 cells) of an autoclaved *Escherichia coli* (K-12 strain) suspension, 5 days before cell harvesting (activated cells). Macrophages from triamcinolone-treated rats were obtained 20 h after intramuscular injection of 125 μl of triamcinolone (7.5 mg/kg body wt.) [12].

Abbreviations used: Fru-2,6-P_2, fructose 2,6-bisphosphate; Fru-6-P, fructose 6-phosphate; PFK-1, 6-phosphofructo-1-kinase (EC 2.7.1.11); PFK-2, 6-phosphofructo-2-kinase (EC 2.7.1.105); PMA, phorbol 12-myristate 13-acetate.

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Table 1. Effect of macrophage activation on Fru-2,6-P_2 content, lactate release and PFK-1 and PFK-2 activities

Resident and activated macrophages were preincubated for 1 h and further incubated in 10 mM-glucose/KRB-Hepes for 1 h in the absence or presence of 100 nM-PMA (Expt. 1). In another experiment (Expt. 2) PFK-1 (maximal activity) and PFK-2 activities were assayed in resident or activated macrophages which had not been incubated. The values are means ± S.E.M. for three separate experiments: [a]$P < 0.01$ for comparison with control cells (no additions); [b]$P < 0.01$ for comparison with resident cells.

Expt. no.	Status of cells	Treatment	Fru-2,6-P_2 (pmol/mg of protein)	Lactate (nmol/mg of protein)	Enzyme activity: PFK-1 (m-units/mg of protein)	Enzyme activity: PFK-2 (μ-units/mg of protein)
1	Resident	None	2.4±0.1	116±3	–	–
		PMA	3.2±0.1[a]	129±5[a]	–	–
	Activated	None	9.1±0.3[b]	197±5[b]	–	–
		PMA	14.9±0.2[a,b]	236±6[a,b]	–	–
2	Resident	Non-incubated	–	–	13±0.6	2.0±0.2
	Activated	Non-incubated	–	–	61±0.8[b]	6.6±0.3[b]

Harvest of macrophages and cell incubations

Peritoneal macrophages were harvested and purified by adherence to plastic as previously described [13]. By this method, as previously reported, the purity of the cell preparations are about 90–95% for resident [14] and activated [15] macrophages. The cells were washed in oxygenated Krebs–Ringer bicarbonate buffered with 10 mM-Hepes, pH 7.4 (KRB-Hepes). This preparation of macrophages was preincubated in KRB-Hepes without additions for 1 h. The cells were further incubated in 10 mM-glucose/KRB-Hepes with the additions indicated in the Tables and Figures. The same amount of solvent was added to control incubations, and it was verified that parameters under study were not affected.

Enzyme and metabolite assays

Fru-2,6-P_2 content was assayed in alkaline extract as in [7] according to the original method of Van Schaftingen *et al.* [16]. Medium lactate was assayed as in [7]. Protein was measured by a modification [17] of the Lowry method.

For the determination of PFK-1 and PFK-2 activities, the cell suspension was sonicated in 50 vol. (v/w) of 20 mM-Hepes/50 mM-KCl/2 mM-EDTA/5 mM-$MgCl_2$/1 mM-dithiothreitol at pH 7.5. The enzymes were routinely partially purified with poly(ethylene glycol) as in [18].

Assays of PFK-1 were performed under conditions of maximal activity at pH 7.8 in the presence of 5 mM-fructose 6-phosphate (Fru-6-*P*), 5 mM-KH_2PO_4, 1.25 mM-ATP, 1.5 mM-ATP and 5 mM-$MgCl_2$ [19]. For kinetic studies, PFK-1 was assayed under sub-optimal conditions as in [19] at pH 7.0 in the presence of 2.5 mM-ATP, as well as with substrates and effectors at the concentrations indicated in the Figures.

PFK-2 was measured by the formation of Fru-2,6-P_2 as in [20]. Briefly, assays were performed in a total volume of 0.1 ml of 100 mM-KCl/5 mM-ATP/5 mM-KH_2PO_4/2 mM-Fru-6-*P*/5 mM-$MgCl_2$ buffered at pH 7.1 with 100 mM-Hepes. The reaction was carried out at 30 °C and stopped after 10 min by addition of 0.1 ml of 100 mM-NaOH. Fru-2,6-P_2 concentration was measured as described above.

Materials

Fru-2,6-P_2 standard was kindly donated by E. Van Schaftingen and H. G. Hers (Laboratorie de Chimie Physiologique, Brussels, Belgium). Triamcinolone acetonide was injected as the pharmaceutical preparation Trigon Depot (Squibb). All biochemicals and purified enzymes were obtained from Sigma and Boehringer.

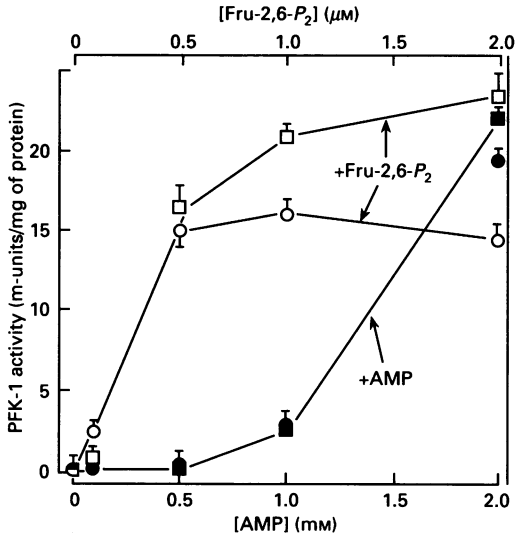

Fig. 1. Effect of Fru-2,6-P_2 and AMP on PFK-1 activity in resident (○, ●) and activated (□, ■) macrophages

PFK-1 was assayed in the presence of Fru-2,6-P_2 (○, □) or AMP (●, ■). PFK-1 assays were performed under sub-optimal conditions at pH 7.0 with 2.5 mM-ATP and 0.1 mM-Fru-6-*P*. The values are means ± S.E.M. for three separate experiments.

RESULTS

Fru-2,6-P_2 and enzyme activities in activated macrophages

The content of Fru-2,6-P_2 in activated macrophages incubated with 10 mM-glucose was greatly increased with respect to resident macrophages (Table 1, Expt. 1). Incubation of activated macrophages with 100 nM-phorbol 12-myristate 13-acetate (PMA) increased Fru-2,6-P_2 to values 1.6-fold greater than in control cells. The response to PMA of resident macrophages was less (1.3-fold increase) that for activated cells. Although PMA significantly increased lactate release in both resident and activated cells, the increase was less than that in Fru-2,6-P_2 content. Other experiments were undertaken to analyse the basal activities of PFK-1 and PFK-2 in both non-incubated resident and activated macrophages (Table 1, Expt. 2). Both enzyme activities were elevated in the activated state. However, the increase in PFK-1 activity was greater (about 5-fold), but did not correlate with the modest increase in lactate output (1.7-fold). The increase in PFK-2 in activated macrophages was less than that in PFK-

Table 2. Interaction between triamcinolone acetonide treatment and the activation state on Fru-2,6-P_2 content, lactate release and PFK-1 and PFK-2 activities in macrophages

Macrophages from non-treated and triamcinolone-treated rats were processed as indicated in Table 1. Absolute values for non-treated macrophages are given in Table 1. The values are means ± S.E.M. for three separate experiments: [a] $P < 0.01$ for comparison with control cells (no additions); [b] $P < 0.01$ for comparison with resident cells.

Expt. no.	Status of cells	Treatment	Inhibition by triamcinolone acetonide (%)			
			Fru-2,6-P_2	Lactate	PFK-1	PFK-2
1	Resident	None	38±2	34±3	–	–
		PMA	49±3[a]	18±2[a]	–	–
	Activated	None	84±4[b]	87±5[b]	–	–
		PMA	88±4[b]	90±4[b]	–	–
2	Resident	Non-incubated	–	–	39±2	45±3
	Activated	Non-incubated	–	–	96±5[b]	76±4[b]

1 and was more closely related to the observed changes in Fru-2,6-P_2 content.

Fig. 1 illustrates the effects of Fru-2,6-P_2 and AMP on the PFK-1 activity from resident and activated macrophages. PFK-1 was more sensitive to stimulation by Fru-2,6-P_2 than to that by AMP, in agreement with previous results in hepatocytes and other cells [21]. It is noteworthy that the kinetic response of PFK-1 to AMP was similar in both preparations of enzyme (from resident and activated macrophages), whereas Fru-2,6-P_2 produced a small but significant increase in the activity of enzyme from activated cells. Therefore the activation state caused a modification in the glycolytic flux at the PFK-1 step, which could be attributed partially to the increase of responsiveness of PFK-1 to Fru-2,6-P_2. Data shown in Table 1 indicate that the activated state was accompanied by an increase in Fru-2,6-P_2 content of nearly 4-fold, and that lactate release was doubled. Therefore this increase in Fru-2,6-P_2 content could explain the stimulation of glycolysis in conjunction with the increase in PFK-1 activity.

Effect of triamcinolone acetonide on PFK-1 and PFK-2 activities in activated macrophages

Intraperitoneal injection of the artificial glucocorticoid, triamcinolone acetonide, to both normal and *E. coli*-treated rats caused a significant decrease in Fru-2,6-P_2 content and lactate release when the cells were incubated with glucose or glucose plus PMA. PFK-1 and PFK-2 activities were also diminished.

Table 2 shows that the intracellular content of Fru-2,6-P_2 and lactate release were inhibited by about 35% in resident macrophages from triamcinolone-treated rats compared with resident macrophages from non-triamcinolone-treated rats. It is noteworthy that the percentage inhibition of Fru-2,6-P_2 content and lactate release in activated macrophages produced by the triamcinolone-treatment was greater (about 85%) than in resident cells. Triamcinolone treatment induced a similar inhibition of lactate release and Fru-2,6-P_2 content in both resident and activated macrophages incubated with 100 nM-PMA compared with cells incubated with glucose alone.

The analysis of PFK-1 and PFK-2 activities showed that both were decreased in resident and activated macrophages from triamcinolone-treated rats (Table 2). In both states of the cells there was residual enzyme activity after triamcinolone treatment, which could not be abolished by prolonged administration of glucocorticoid. The effect of triamcinolone was also dose- and time-dependent (result not shown), as previously demonstrated in thymus lymphocytes [12]. The present data show that the metabolic processes characteristic of the activated state of

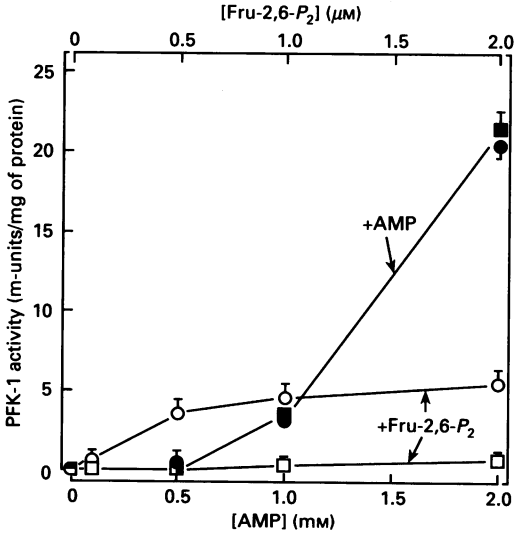

Fig. 2. Effect of Fru-2,6-P_2 and AMP on PFK-1 activity in resident (○, ●) and activated (□, ■) macrophages after triamcinolone acetonide treatment *in vivo*

PFK-1 from triamcinolone-treated rat macrophages was assayed in the presence of Fru-2,6-P_2 (○, □) or AMP (●, ■). PFK-1 assays were performed under sub-optimal conditions at pH 7.0 with 2.5 mM-ATP and 0.1 mM-Fru-6-P. The values are means ± S.E.M. for three separate experiments.

macrophages can be antagonized by glucocorticoid treatment. Further experiments were undertaken to analyse the nature of the modification of PFK-1 produced by triamcinolone treatment. Fig. 2 illustrates the effect of Fru-2,6-P_2 on partially purified PFK-1 from triamcinolone-treated macrophages. Fru-2,6-P_2 did not restore PFK-1 activity to the values observed in non-triamcinolone-treated macrophages. The effects of glucocorticoid treatment were similar in resident and activated cells. However, AMP activated PFK-1 from triamcinolone-treated rat macrophages to values found in control cells. This suggests that triamcinolone treatment somehow alters the Fru-2,6-P_2 domain of PFK-1 protein, without affecting the AMP-binding site.

Effect of other agents

Phagocytic cells have specific receptors for a variety of agents, which can modify their response and metabolic activity [22]. Experiments were undertaken to analyse the changes in Fru-2,6-P_2 content in response to some of these agents (Table 3). Results

Table 3. Effect of different agents on Fru-2,6-P_2 content in resident macrophages

Resident macrophages were preincubated for 1 h and further incubated with 10 mM-glucose/KRB-Hepes and different agents for 1 h. The results are means ± S.E.M. of at least three separate experiments: *$P < 0.01$ for comparison with control cells (no additions).

Additions	Fru-2,6-P_2 (% relative to control)
Thimerosal (10 μM)	150 ± 10*
Concanavalin A (5 μg/ml)	108 ± 2
A23187 (1 μM)	72 ± 5*
Pyruvate (10 mM)	80 ± 5*
Ethanol (1 %, v/v)	66 ± 8*
Dimethyl sulphoxide (0.1 %, v/v)	95 ± 4
Peroxidase (50 units/ml) (with 0.5 mM-glucose)	60 ± 7*
Peroxidase (50 units/ml) (with 10 mM-glucose)	90 ± 7
Insulin (0.1 unit/ml)	103 ± 5
Adrenaline (1 μM)	97 ± 2
Tri-iodothyronine (0.15 μg/ml)	101 ± 3

from this laboratory showed that thimerosal stimulates glycolysis, increases Fru-2,6-P_2 content and mobilizes Ca^{2+} in thymus lymphocytes [23]. Thimerosal had a similar action on Fru-2,6-P_2 content in macrophages (Table 3). Although it is difficult to explain this action, it may reflect a general property of thimerosal to stimulate glucose metabolism. Concanavalin A does not modify the Fru-2,6-P_2 content. By contrast, other compounds produced a different pattern of inhibition. Pyruvate (10 mM) and ethanol (1 %, v/v) markedly decreased the Fru-2,6-P_2 content. These results are in agreement with previous findings in isolated hepatocytes [24] and adipose tissue [25]. An inhibitory effect of Ca^{2+} ionophore A23187 on Fru-2,6-P_2 content was systemically observed. A similar action of the ionophore was observed in thymus lymphocytes (results not shown). However, a stimulating effect of A23187 on Fru-2,6-P_2 content has been described in B-lymphocytes [20], but we have no explanation for this discrepancy. Peroxidase induces tumour-necrosis-factor production and cytotoxicity in macrophages [26]. Its effect on glycolysis was dependent on the concentration of glucose. Thus, at 0.5 mM-glucose, peroxidase decreased Fru-2,6-P_2 content by about 40 % compared with control cells. This inhibitory effect was almost cancelled by 10 mM-glucose. Dimethyl sulphoxide, the solvent vehicle for hydrophobic agents (e.g. phorbol esters, ionophores), did not modify Fru-2,6-P_2 content at concentrations used in the experiments ($\leq$ 0.1 %, v/v). On the other hand, hormones, such as insulin and tri-iodothyronine, which are known to have pronounced effects on carbohydrate metabolism in liver, muscle and adipose tissue, failed to modify Fru-2,6-P_2 content in macrophages. Although macrophages and lymphocytes possess a variety of hormone receptors [27], this might indicate that these cells are mainly regulated by other types of molecules (e.g. lymphokines and regulatory factors). The findings also show that changes in Fru-2,6-P_2 content caused by several agents cannot be correlated with other effects produced in macrophages.

DISCUSSION

It has been shown that the activated state of macrophages implies a greater release of lactate as a consequence of the stimulation of glucose metabolism [6]. However, to the best of our knowledge, few studies have addressed this phenomenon. Previously it has been reported that macrophages utilize glucose and glutamine at high rates and that these cells are characterized by a high activity of glucose-metabolism enzymes [28,29]. Our data show that the Fru-2,6-P_2 content and PFK-1 are increased several-fold in the activated state. Previous studies have shown that Fru-2,6-P_2 content can be used as an index of glycolytic rate under conditions in which ATP levels are maintained. Liver Fru-2,6-P_2 content responds to small variations in glucose concentration *in vivo* compared with lactate release or the production of ^3H_2O from [3-^{3}H]glucose, measured as an index of glycolysis [30]. However, in anoxic hepatocytes [31] and in adrenaline-stimulated adipose tissue [32] changes in Fru-2,6-P_2 content are not related to lactate output. The increased concentration of Fru-2,6-P_2 in activated macrophages might explain how PFK-1 activity, and hence lactate liberation, are increased. In addition to a greater activity of PFK-1 in the activated state, we observed an increase in PFK-2 activity by phorbol ester. Taken together, these results emphasize the importance of glycolysis to supply energy and accessory metabolites for the activated state of macrophages [29].

The inhibitory effect of triamcinolone on the increased glycolytic parameters observed during activation might indicate that many of the therapeutic effects of glucocorticoids in the inflammation are mediated through direct action on macrophages. Glucocorticoids have previously been shown to depress glucose metabolism [9]. This inhibitory effect is rather selective, since no effects of glucocorticoid on hexokinase, glucose 6-phosphate dehydrogenase or pyruvate kinase have been demonstrated [33]. The present data showing the inhibition of Fru-2,6-P_2 content, PFK-1 and PFK-2 activities in triamcinolone-treated rats agree with previous results showing a correlation between inhibition of macrophage activation and glucocorticoid action [11]. This correlation is based on the observation that glucocorticoids specifically inhibit or reverse biochemical functions which had been enhanced in the activated state. Similar evidence suggests that, *in vitro*, glucocorticoids affect only the induced functions and do not affect the 'constitutive' processes of these cells [34]. The effects of glucocorticoids are mediated through binding to specific receptor proteins located in the cytoplasm of target cells. The interaction of the steroid-receptor complex with chromatin modulates gene expression to produce different responses. Studies with glucocorticoids at the nuclear level in thymocytes and phagocytic cells have shown that the activation of nucleases is responsible for cells lysis [35]. By contrast, glucocorticoids exhibit a permissive effect in other cells by stimulating carbohydrate metabolism (e.g. in liver) [36]. The tissue-specific effect of glucocorticoids is clearly demonstrated in the recent work of Marker *et al.*, where triamcinolone treatment restored the mRNA encoding rat hepatic PFK-2/fructose-2,6-bisphosphatase together with the enzyme concentration [37]. These data contrast with the inhibition of glucose utilization by phagocytic cells [11], the inhibition of Fru-2,6-P_2 content in thymus lymphocytes [12], and with the present results on Fru-2,6-P_2 content and PFK-1 and PFK-2 activities. It is difficult to interpret these differences. The effect of glucocorticoids in phagocytic cells might be due to the decreased transcription of the genes coding for PFK-1 and PFK-2. In addition, inhibition of PFK-1 and PFK-2 by glucocorticoids might have been due to the decreased production of several lymphokines, including interleukin-1 [38] and interleukin-2 [39], which have been shown to increase Fru-2,6-P_2 content [40].

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