Control of fructose 2,6-bisphosphate levels in rat macrophages by glucose and phorbol ester

R. Bustos and F. Sobrino

Departamento de Bioquímica Médica y Biología Molecular, Facultad de Medicina, Avda. Sanchez Pizjuan, 4, Universidad de Sevilla, Sevilla 41009, Spain

The presence of fructose 2,6-bisphosphate (Fru 2,6-P_2) in elicited peritoneal macrophages of rat was examined. These cells possess an active phosphofructokinase-2 which is diminished by citrate and only slightly inhibited by glycerol 3-phosphate. Phosphofructokinase-1 submaximal activity was increased 26-fold by the addition of 1 μM Fru 2,6-P_2. Incubation of cells without glucose decreased the amount of Fru 2,6-P_2 to zero, but further addition of 5 mM glucose increased the levels of the sugar ester 20-fold. In addition, the presence of phorbol ester potentiated the synthesis of Fru 2,6-P_2. By contrast phenylisopropyladenosine or prostaglandin $F_{2\alpha}$ inhibited the production of Fru 2,6-P_2.

Fructose 2,6-bisphosphate; Lactate; Macrophage; (Peritoneal cell)

1. INTRODUCTION

Macrophages undergo a respiratory burst upon phagocytic stimulation or exposure to the soluble agonist phorbol ester or *cis*-polyunsaturated acids [1–4]. The biochemical sequence of this stimulation is likely to involve the activation of multiple pathways. One of these is the pentose phosphate shunt in order to provide NADPH for NADPH-oxidase [5]. Recently, it has been shown that elicited macrophages possess high levels of key enzymes of the pentose phosphate shunt [6]. The activity of glucose 6-phosphate dehydrogenase in mouse macrophages is almost 8-fold higher than that found in mouse liver, and these cells also possess high activities of 6-phosphogluconate dehydrogenase, hexokinase and 6-phosphofructokinase. It has been suggested that the glycolytic pathway is enhanced during phagocytosis [7].

The present study investigates whether fructose 2,6-bisphophshate (Fru 2,6-P_2), a potent stimulator of phosphofructokinase-1 in many cells [8,9], is present in macrophage cells, and, in addition, whether it plays a role in regulating the glycolytic flux when these cells are incubated with different types of stimulators. In this work it is shown that Fru 2,6-P_2 is present in macrophages and other peritoneal cells at similar levels to those found in liver and that its concentration is modified during cell activation.

2. MATERIALS AND METHODS

Elicited macrophages were obtained from the peritoneal cavity of male Wistar rats 3 days after injection of 5 ml of 6% casein in 0.9% NaCl as in [10]. The cells were washed in Krebs Ringer bicarbonate (KRB) [11] buffered with 10 mM Hepes, pH 7.4, and oxygenated with O_2/CO_2 (95:5). The cells were maintained in an incubator at 37°C in air/CO_2 (19:1) in fresh KRB-Hepes supplemented with 2% (w/v) bovine serum albumin and 10 mM glucose. After 2 h of incubation the cell-culture dishes were washed three times with cold KRB-Hepes to remove non-adherent cells. In some experiments these supernatants were centrifuged and the pellet of cells suspended in fresh buffer KRB-Hepes. The adherent macrophages were gently separated with a rubber policeman and suspended in KRB-Hepes without additions unless otherwise indicated. The sum of

Correspondence address: F. Sobrino, Dpto de Bioquímica Médica y Biología Molecular, Facultad de Medicina, Avda. Sanchez Pizjuan, 4, Universidad de Sevilla, Sevilla 41009, Spain

adherent plus non-adherent cells is referred to in the text as total peritoneal cells. This preparation of macrophages was incubated in KRB-Hepes without additions for 1 h (preincubated cells) in order to deplete intracellular glycogen and to obtain low levels of Fru 2,6-P$_2$ and lactate. The cells were then incubated with the additions indicated in the text. Fru 2,6-P$_2$ was extracted and measured as in [12]. Medium lactate was assayed as in [13] and glycogen as in [14]. Phosphofructokinase-1 [15] and phosphofructokinase-2 [16] activities were analyzed in preparations partially purified with polyethylene glycol by the standard methods. The level of protein was analyzed by a modified Lowry method [17]. Fru 2,6-P$_2$ standard was kindly donated by E.V. Schaftingen and H.G. Hers (Laboratoire de Chimie Physiologique, Brussels, Belgium). All biochemical and purified enzymes were obtained from Sigma and Boehringer.

3. RESULTS AND DISCUSSION

3.1. Characterization of fructose 2,6-bisphosphate enzymatic system

Fig.1 shows the time course of intracellular levels of Fru 2,6-P$_2$ and the concentration of lactate in the medium in elicited total peritoneal cells incubated without additions. For the untreated cells the total amount of Fru 2,6-P$_2$ was about 70 pmol/mg protein, which represents a value similar to that found in many cells (review [18]). Over 30–60 min of incubation this value declined to practically zero. At this time, an increase in the concentration of lactate released to medium was observed. The analysis of the glycogen levels in these conditions reveals that in non-incubated cells there was 22.1 μg/mg protein, and this value decreased to 2.5 μg/mg protein after 90 min. This indicates that the high levels of Fru 2,6-P$_2$ observed at the beginning of incubation time (and presumably of other glycolytic intermediates) was sustained by the degradation of glycogen. By contrast, in preincubated cells (e.g. with low levels of glycogen) the concentration of Fru 2,6-P$_2$ was also low. A correlation between both parameters (Fru 2,6-P$_2$ and glycogen) was described in the adipose tissue [19]. The analysis of partially purified PFK-1/PFK-2 system in untreated macrophages gave the following results: (i) the incubation of PFK-2 with 1.6 μg/0.2 ml catalytic subunit of cAMP-dependent protein kinase and 0.5 mM MgATP did not modify its activity at either pH 6.6 (active form) or pH 8.5 (total activity) [16] (not shown); (ii) PFK-2 activity was inhibited about 73% by 0.5 mM citrate, while a lack of effect was observed with glycerol 3-P. These properties
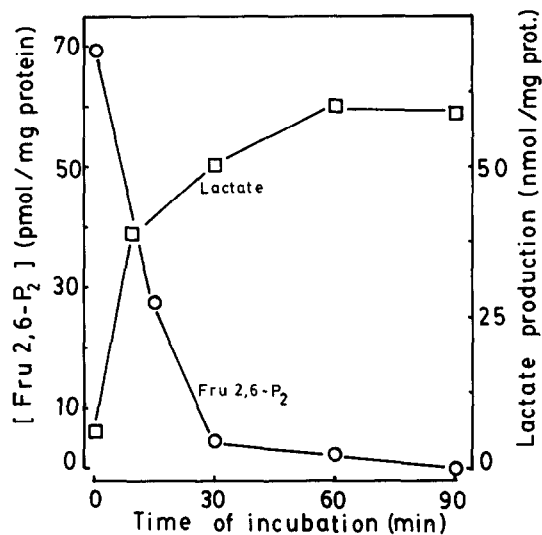

Fig.1. Levels of Fru 2,6-P$_2$ (○) and lactate (□) in total peritoneal cells incubated without additions. Values shown are the means of two separate experiments.

resemble those of the cardiac isoenzyme of PFK-2 [20]; and (iii) the activity of PFK-1 assayed under submaximal conditions (2.5 mM ATP and 0.1 mM Fru 6-P at pH 7.0) was stimulated about 26-fold after the addition of 1 μM Fru 2,6-P$_2$.

The effect of medium glucose concentration on Fru 2,6-P$_2$ concentration in preincubated or non-preincubated macrophages was studied in the next experiments. Maximal values were found at 5 mM glucose. Higher levels of the sugar did not increase the concentration of Fru 2,6-P$_2$. The apparent $K_{0.5}$ for glucose was about 0.5 mM in preincubated cells. Under this condition, it is noteworthy that 5 mM glucose produced a 20-fold increase in the concentration of Fru 2,6-P$_2$ as compared to control cells. It was also observed that in non-preincubated macrophages the initial amount of Fru 2,6-P$_2$ was higher than in 1 h preincubated cells. However, at 5 mM glucose maximal values of Fru 2,6-P$_2$ were similar in both conditions. The release of lactate followed a profile like that of Fru 2,6-P$_2$ synthesis (not shown).

3.2. Effect of phorbol ester

Fig.2 shows the effect of PMA on Fru 2,6-P$_2$ levels in purified macrophages incubated at different concentrations of glucose. PMA enhanced the effect of glucose to increase the Fru 2,6-P$_2$ con-
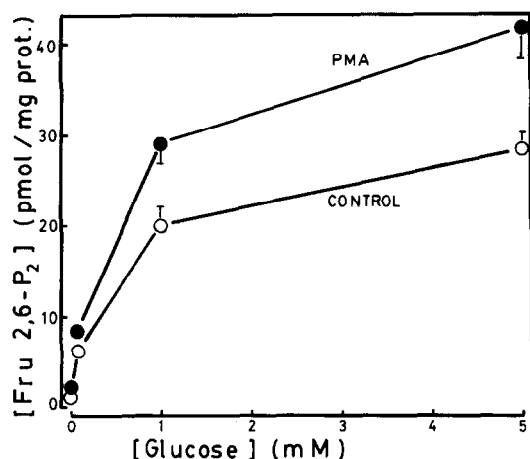
Fig.2. Effect of PMA on Fru 2,6-P_2 concentration in macrophages incubated with various concentrations of glucose. Previously the cells were preincubated without additions. The concentration of Fru 2,6-P_2 was measured in cells incubated for 1 h with (●) or without (○) 100 nM PMA. Values shown are the means ± SE for four separate experiments.

Table 1

Fructose 2,6-bisphosphate concentration of macrophages incubated with various agonists

Additions	Fru 2,6-P_2 (pmol/mg protein)
Control	28.25 ± 1.1
PIA (50 μM)	21.38 ± 0.8*
PGF$_{2\alpha}$ (50 μM)	17.73 ± 0.9*
ADP/ATP (50/100 μM)	27.99 ± 1.2
Peroxidase (50 U/ml)	27.83 ± 1.0
NaIO$_4$ (0.5 nM)	26.72 ± 1.3

Purified macrophages were incubated without additions for 1 h. The cells were washed and further incubated with 10 mM glucose and the following additions for 1 h: PIA, phenylisopropyladenosine; prostaglandin F$_{2\alpha}$; ADP/ATP; peroxidase and Na periodate. Values shown are means ± SE for three separate experiments. * $P < 0.01$ vs control

centration. At 5 mM glucose, which produces a maximal amount of Fru 2,6-P_2, the stimulation by PMA reached about 50% over the control cells. By contrast to this effect, the addition of PMA to macrophages was not followed by an increase in the release of lactate and this fact was independent of whether the cells were preincubated or not. Although it is not possible to give a definitive explanation about this fact, it could be suggested that the occurrence of recycling of glycolytic intermediates after the phosphofructokinase-1 step. Moreover, this seems to be particularly the case in macrophages, since in other peritoneal cells, non-macrophages, PMA affected both parameters (e.g. Fru 2,6-P_2 and lactate levels) (not shown).

3.3. Effect of other agonists

The following experiments were addressed to analyze in purified macrophages the effect of several agents known to alter some specific functions of phagocyting cells [21]. The cells were preincubated without additions and further incubated in the presence of 10 mM glucose and the indicated agonists for 1 h. Under some of these conditions, as e.g. after phenylisopropyladenosine or prostaglandin F$_{2\alpha}$, the concentrations of Fru 2,6-P_2 are significantly reduced (table 1). These latter agents cause an increase in intracellular cyclic-

AMP levels in neutrophils [22] and in monocytes [23]. However, this does not explain the decrease of Fru 2,6-P_2 due to the lack of inhibition by phosphorylation (cyclic-AMP dependent) of PFK-2 from macrophages. By contrast the presence of ADP/ATP, peroxidase and NaIO$_4$ did not modify the levels of Fru 2,6-P_2. The same result was found with insulin (not shown). On the other hand, the addition of digitonin (10 μg/ml) which enhances the production of anion superoxide [24] killed the cells after 1 h of incubation, and therefore rendered the measurement of Fru 2,6-P_2 inappropriate.

4. CONCLUSIONS

Fructose 2,6-bisphosphate is a potent activator of phosphofructokinase-1 obtained from a variety of tissues. In normal conditions, the role of Fru 2,6-P_2 in controlling the rates of glycolysis and gluconeogenesis is well accepted, although some doubts arose when the glycolysis was stimulated by anoxia in isolated hepatocytes [25]. The present results demonstrate that Fru 2,6-P_2 is present in non-stimulated macrophages, and that the levels of Fru 2,6-P_2 can be increased about 20-fold over control by glucose. Our data also show that the concentrations of Fru 2,6-P_2 are modulated by factors such as PMA, phenylisopropyladenosine, prostaglandin F$_{2\alpha}$ or by the removal of glucose from external medium. In order to analyze

145

whether Fru 2,6-P_2 levels may be taken as an index of glycolytic flux in macrophages, the relationship between its values and the amount of released lactate, obtained in the same experiment, was calculated. There is an acceptable correlation index ($r = 0.883$) between both parameters. This suggests that Fru 2,6-P_2 regulates the glycolytic flux in the cells by the activation of PFK-1, which is consistent with the observation that Fru 2,6-P_2 increased the activity in a purified preparation of PFK-1 from macrophages.

The positive effect of glucose and PMA was also evident in peritoneal cells other than macrophages. The amount of macrophages represent approx. 90% of the total number of peritoneal cells. The other peritoneal cells are mainly composed of lymphocytes and polymorphonuclear leukocytes [26]. The occurrence of Fru 2,6-P_2 in lymphocytes B was previously demonstrated, and also its modulation by PMA [16].

REFERENCES

[1] Babior, B.M. (1978) N. Engl. J. Med. 298, 659–668.
[2] De Chatelet, L.R., Shirley, P.S. and Johnston, R.B., jr (1976) Blood 47, 547–554.
[3] Pick, E. and Keisari, Y. (1981) Cell Immunol. 59, 301–318.
[4] Segal, A.W., Garcia, R., Goldstone, A.H., Cross, A.R. and Jones, O.T.G. (1981) Biochem. J. 196, 363–367.
[5] Sbarra, A.J. and Karnovsky, M.L. (1959) J. Biol. Chem. 234, 1355–1362.
[6] Newsholme, P., Curi, R., Gordon, S. and Newsholme, E.A. (1986) Biochem. J. 239, 121–125.
[7] Roos, D. and Van der Stijl-Neijenhuis, J.S. (1980) in: Mononuclear Phagocytes (Van Furth, ed.) pp.1119–1211, Martinus Nijhoff Publishers, Den Haag.
[8] Hers, H.G. and Van Schaftingen, E. (1982) Biochem. J. 206, 1–12.
[9] Pilkis, S.J., El-Maghrabi, M.R., McGrane, M., Pilkis, J., Fox, E. and Claus, T.H. (1982) Mol. Cell. Endocrinol. 25, 245–266.
[10] Mosier, D.E. (1984) Methods Enzymol. 108, 294–297.
[11] Krebs, H.A. and Henseleit, K. (1932) Hoppe Seylers Z. Physiol. Chem. 210, 33–66.
[12] Van Schaftingen, E. (1984) in: Methods of Enzymatic Analysis (Bermayer, H.U. ed.) pp.335–341, Verlag Chemie, Weinheim.
[13] Hohorst, H.J. (1963) in: Methods of Enzymatic Analysis (Bermayer, H.U. ed.) pp.266–270, Academic Press, New York.
[14] Sobrino, F. and Hers, H.G. (1980) Eur. J. Biochem. 109, 239–246.
[15] Gualberto, A., Molinero, P. and Sobrino, F. (1987) Biochem. J. 244, 137–142.
[16] Bosca, L., Mojena, M., Diaz-Guerra, M.J. and Marquez, C. (1988) Eur. J. Biochem. 175, 317–323.
[17] Markwell, M.A.K., Haas, S.M., Bieber, L.L. and Tulbert, N.E. (1978) Anal. Biochem. 87, 206–210.
[18] Hue, L. and Rider, M.H. (1987) Biochem. J. 245, 313–324.
[19] Sobrino, F. and Gualberto, A. (1985) FEBS Lett. 182, 327–330.
[20] Rider, M.H. and Hue, L. (1986) Biochem. J. 240, 57–61.
[21] Klebanoff, S.V. and Clark, R.A. (1978) in: The Neutrophil: Function and Clinical Disorders, pp.283–286, Elsevier/North-Holland, Amsterdam.
[22] Cox, J.P. and Karnovsky, M.L. (1973) J. Cell. Biol. 59, 480–490.
[23] Lappin, D. and Whaley, K. (1984) Clin. Exp. Immunol. 57, 454–460.
[24] Cohen, H.J. and Chovaniec, M.E. (1978) J. Clin. Invest. 61, 1088–1096.
[25] Hue, L. (1982) Biochem. J. 206, 359–365.
[26] Tsunawaki, S. and Nathan, C.F. (1984) J. Biol. Chem. 259, 4305–4312.