Synergistic activation of 2-deoxy-D-glucose uptake in rat and murine peritoneal macrophages by human macrophage colony-stimulating factor-stimulated coupling between transport and hexokinase activity and phorbol-dependent stimulation of pentose phosphate-shunt activity

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1. Transport and accumulation of 2-deoxy-D-glucose (2dGlc) in rat and murine peritoneal macrophages were investigated by using C-1-³H-labelled and C-2,6-³H-labelled 2dGlc. 2. There was active accumulation of both C-1- and C-2,6-labelled 2dGlc by quiescent rat and murine macrophages via a phloretin-inhibitable transport system. 3. The rate of uptake and accumulation of 2dGlc (C-1 label) was increased by exposure to human macrophage colony-stimulating factor (mCSF-1) (1000 units/ml) in both murine and rat macrophages. This indicates that mCSF-1 enhances coupling between hexokinase activity and glucose transport at the endofacial surface of the transporter. 4. Phorbol 12-myristate 13-acetate ('phorbol') at 40 nM stimulated 2dGlc in rat macrophages entirely by increasing the C-2,6 label uptake. This indicates that phorbol stimulates 2dGlc uptake mainly by increasing the activity of the pentose phosphate pathway. 5. Simultaneous exposure to phorbol and mCSF-1 stimulates 2dGlc uptake to a greater extent than found with either phorbol or mCSF-1 alone. This result is explained by a simultaneous enhancement of pentose phosphate-pathway activity and of hexokinase activity acting at the endofacial surface of the cell membrane. The dual activation of these serial processes coupled to the loss of the reaction products of the pentose phosphate-shunt pathway from the cells in the form of reactive oxygen intermediates, protons and CO₂, could explain the synergistic action of phorbol and mCSF-1 in activation of sugar transport in macrophages.

INTRODUCTION

Activation of rat or mouse peritoneal macrophages and other phagocytic cells involves an increase in oxygen utilization with H₂O₂ and reactive-oxygen-intermediate (ROI) production (Badway & Karnovsky, 1980). Additionally glucose transport and metabolism via the pentose phosphate pathway are increased (Arthur *et al.*, 1986, 1988).

There are a number of activators of macrophages, among which are bacterial antigens, specific growth factors, e.g. colony-stimulating factor (mCSF-1) (Walker *et al.*, 1985; Hamilton *et al.*, 1986, 1988), interleukin-3 (IL-3) (Whetton *et al.*, 1984) and phorbol esters (Hamilton *et al.*, 1988), and also calcitriol (Segawa *et al.*, 1988) and retinoic acid (Miyaura *et al.*, 1986), which are known to promote growth and mitosis of mouse macrophages in culture.

Since macrophage production of ROI is determined by oxidation of NADPH via a membrane-bound flavoprotein (Babior & Peters, 1981; Gabig, 1983) and NADPH is generated when glucose is transformed to ribose 5-phosphate via the pentose phosphate pathway (2 mol of NADPH/mol of ribose 5-phosphate produced; Landau & Wood, 1983), it seems probable that production of ROI is linked to the increased pentose

phosphate-pathway activity. A direct correlation between ROI production and pentose phosphate-pathway activity has been observed in hepatic macrophages (Arthur *et al.*, 1986), and recently it has been suggested that some link may exist between glucose transport or metabolism and ROI production in human neutrophils, as high concentrations of mannose inhibit ROI production and pentose phosphate-pathway activity (Rest *et al.*, 1988).

Active transport of sugar

2-Deoxy-D-glucose (2dGlc) uptake by rat thymocytes is linked to hexokinase activity acting at the endofacial surface of the glucose transporter (Hume & Weidemann, 1980; Naftalin & Smith, 1987; Naftalin & Rist, 1989). Hexokinase activity depletes permeant sugar from the endofacial surface of the transporter and thereby accelerates the unidirectional rate of sugar uptake. Hexokinase activity adjacent to the endofacial surface of the transporter prevents escape of intracellular free sugar from rat thymocytes and Chinese-hamster ovary cells (Naftalin & Rist, 1989; Faik *et al.*, 1989). Hence a significant rate of breakdown of 2dGlc6P to 2dGlc within the cytosol, together with coupling between hexokinase and glucose transport, will lead to active accumulation of free sugar within the cytosol.

Abbreviations used: mCSF-1, human macrophage colony-stimulating factor; 2dGlc6P, 2-deoxy-D-glucose 6-phosphate; ROI, reactive oxygen intermediate; phorbol, phorbol 12-myristate 13-acetate.

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Phorbol-dependent activation of 2dGlc uptake in rat thymocytes arises from an increased degree of coupling between the hexokinase reaction and transport. This reduces the slippage between transport and hexokinase activity and will lead to (i) an increased rate of 2dGlc uptake; (ii) an increase in the rate of uptake of sugar label from the external solution into the hexose phosphate pool; (iii) an increase in free sugar accumulation within the cytosol; and (iv) a reduction in free sugar exit and a reduction in the K_m for net uptake of sugar. There are several possible ways in which the degree of coupling can be altered, e.g. attachment of hexokinase to the endofacial surface of the glucose transporter following acylation of the enzyme (Tower *et al.*, 1988).

Synergism between stimulants to activation

Human macrophage colony-stimulating factor (mCSF-1) and interleukin-3 (IL-3) act synergistically to promote haematopoiesis in primates (Donahue *et al.*, 1988). Synergism has also been demonstrated between phorbol 12-myristate 13-acetate (phorbol) and CSF-1 in enhancement of 2dGlc uptake by murine macrophages (Hamilton *et al.*, 1988).

Since it is apparent that the pentose phosphate pathway is closely linked to glucose-transport activity in macrophages and synergistic activation of macrophages may involve more than one kind of receptor-activated response, we were curious to know whether phorbol and mCSF-1 function independently, or together, in enhancing sugar transport via the pentose phosphate pathway in macrophages, and also whether these agents affect active accumulation of 2dGlc in macrophages in a similar way to that seen with rat thymocytes (Naftalin & Rist, 1989).

Answers to these questions can be obtained by adapting the differential-label methods for investigating the pentose phosphate pathway to those previously used in our laboratory to study active sugar transport (Arthur *et al.*, 1986; Rest *et al.*, 1988).

METHODS

Quiescent peritoneal macrophages were obtained from Wistar rats (100–150 g body wt.). A 10 ml portion of Hepes (5 mM)-buffered saline (NaCl, 140 mM; KCl, 5 mM; $MgCl_2$, 1.2 mM), pH 7.3, containing heparin (50 units/ml), was injected intraperitoneally into rats, which had been killed by cervical dislocation. The abdominal contents were massaged for 5 min before the peritoneal washings were removed. Three or four rats were used in each experiment with a yield of approx. 2×10^7 cells/rat. With practice, contamination of the peritoneal washings with erythrocytes could be avoided.

Murine macrophages were obtained from albino mice by using Hepes/heparin intraperitoneal injections of Hepes/heparin buffer (4 ml/mouse) as described for rats. The cell yield per mouse was approx. 4×10^6 cells. The cells were washed twice in iso-osmotic Hepes-buffered saline by centrifugation at 1200 *g* for 3 min and resuspension in fresh buffer. This cell suspension, without further modification, was used to estimate sugar transport.

Thymocytes

The method of preparation of rat thymocytes was as described previously (Naftalin & Rist, 1989). Thymuses

were removed by blunt dissection and immersed in Hepes-buffered saline at 20 °C. The tissue was chopped into small pieces and disaggregated in about 50 ml of saline by repeated suction into, and extrusion from, the nozzle of a 10 ml plastic syringe (18–20 cycles). Any floating debris was removed. The cell suspensions were lightly centrifuged at 400 *g* for 1 min to precipitate any heavy aggregates, and the supernatants were decanted and re-centrifuged for 3 min at 2000 *g* in a bench centrifuge to concentrate the cells.

Microscopy and cell counting

The method of obtaining disaggregated thymocytes and of preparation of macrophages does not cause discernible damage to the appearance or integrity of the cells. There is very little cell debris in the preparations, and it is hard to detect any dead cells by using Evan's Blue uptake.

All sugar-uptake rates and estimates of intracellular concentrations of free sugar and hexose phosphates are normalized to 10^{10} cells. As the mean cell volume is approx. 100 fl, 10^{10} cells $\approx$ 1 ml of cell water for both rat and mouse macrophages and rat thymocytes. This was confirmed by measurement of the 3-*O*-methyl-D-glucose space (= 1.3 ml/ 10^{10} rat cells). The cell numbers were estimated by using a model B Coulter Counter. The concentration of cells in the suspension used for influx was approx. 5×10^6 cells/ml. Differential counts of stained preparations of rat macrophages showed more than 90 % macrophages and monocytes.

Activation with phorbol

Before measuring uptake, the cell suspensions at $(2-5) \times 10^6$ cells/ml were preincubated for a period of 30–60 min in solutions containing phorbol (40 nM) or in control solutions. This concentration of phorbol ester was found to give optimal activation over a prolonged incubation. However, in the series of experiments in Tables 3 and 4 (below), where the additive effects of phorbol and mCSF-1 were tested, the cells were preincubated for 1 h with mCSF-1 and only then was phorbol added alone, or to suspensions preincubated with mCSF-1. Thus the cells were exposed to phorbol only during the period of incubation with label, 15 min with rat macrophages or 30 min with mouse macrophages (Tables 3 and 4). This was done to minimize cytotoxic effects resulting from secretion of ROIs.

Activation with macrophage colony-stimulating factor (mCSF-1)

Pure recombinant mCSF-1 (Cetus Corp) was used at a final concentration of 1000 units/ml. The cells were incubated for 60 min with mCSF-1 before measurements of sugar uptake. This concentration of mCSF-1 and preincubation time had been previously shown to stimulate murine macrophages (Boocock *et al.*, 1989) and was also used here for rats.

Radioactive sugar uptake studies

Sugar uptake into the cells was measured using 2-deoxy-D-[C-1- ^3H]- or -[C-2,6- ^3H]-glucose (Amersham International; initial specific radioactivity 11.5 Ci/mmol at a final radioisotope concentration of 0.2 μ Ci/ml). After incubation, sugar uptakes were halted by adding iso-osmotic ice-cold stopping solution to the cell suspensions. This contained enough phloretin and $HgCl_2$ to give final

concentrations of $100\ \mu\text{M}$ and $1\ \mu\text{M}$ respectively. The background passive uptake of sugar into both murine and rat cells was determined by measuring uptake in the presence of stopping solution. All uptake rates are expressed as phloretin-sensitive uptakes. The cell suspensions were centrifuged in a bench centrifuge for 2 min at 3000 *g*. The supernatants were removed, and a sample was retained for the determination of extracellular radioactivity. Then the cells were washed twice more by suspension and re-centrifugation in 4 ml of stopping solution to remove extracellular radioisotope. Finally, the cell pellets were lysed in 4 ml of ice-cold distilled water. This lysate was used to determine the total uptake, free sugar and hexose phosphate concentrations within the cell pellet. Both macrophages and thymocytes were treated in the same way.

The rates of uptake were determined from 2 min to 3 h. The initial rate was linear over the first 15 min in rat macrophages and for 60 min in mouse macrophages.

Estimation of non-phosphorylated and phosphorylated sugar

A Whatman DE81 2.3 cm-diameter filter was washed with 2 ml of ice-cold glass-distilled water by suction via a Buchner filtration tube; then 1 ml of ice-cold cell lysate from approx. $(1-2) \times 10^6$ cells was filtered and the filtrate retained; the filter was washed with a further 2 ml of cold distilled water and this secondary wash filtrate was added to the primary filtrate. The combined filtrate was sampled for free sugar and the remainder rejected. The hexose phosphate retained on the filter was then eluted by washing with 2 ml of 0.5 M-HCl at 20 °C, and a sample of this filtrate was counted for radioactivity to estimate hexose phosphates. Estimates of total, free sugar and hexose phosphates/ 10^{10} cells were obtained after correc-

tion for quench and dilution. The samples were counted for radioactivity in scintillation fluid with the following composition: 500 ml of toluene, 500 ml of Synperonic N (Durham Chemicals Distributors Ltd., Birtley, Chester-le-Street, Co. Durham, U.K.) and 2.5 g of 2,5 diphenyloxazole (Sigma Chemical Co., Poole, Dorset, U.K.).

The efficiency of the separation process was continuously checked by determining whether the sum of the recoveries of free sugar and sugar phosphates was equal to the separately determined total sugar. The method was also checked by using a cell-free system in which D-[^3H]glucose 6-phosphate was generated from labelled D-glucose and ATP catalysed by beef hexokinase obtained from Sigma. The method has been previously shown to give accurate separation of free sugar from hexose phosphates using t.l.c. (Naftalin & Rist, 1989).

Determination of pentose phosphate-pathway activity

In cells such as macrophages, where there is a significant pentose phosphate pathway (Newsholme *et al.*, 1986, 1987), 2dGlc6P is metabolized to 2-deoxyribose 5-phosphate with loss of ^3H - ^{14}C -OH from the 1-position of 2dGlc6P. Hence ^{14}C or ^3H label in the 1 position of 2dGlc will be lost from the cell as $^{14}\text{CO}_2$ or $^3\text{H}_2\text{O}$, whereas label at the other positions of the original 2dGlc will be retained within the cytosol. Thus a marked difference in uptake of label from C-1 or C-6 positions of 2dGlc due to depletion of intracellular C-1 label indicates an active pentose phosphate pathway (Scheme 1).

In cells where 2dGlc is actively accumulated, providing no pentose phosphate pathway is present, it would be expected that an equal rate of uptake and accumulation of [C-1- ^3H]2dGlc and [C-2,6- ^3H]2dGlc will be observed. If there is stimulation of pentose phosphate-pathway activity alone, then the [C-1- ^3H]2dGlc within the cell will

Scheme 1. Transformations of ^3H (C-1 and C-2,6)-labelled 2dGlc via the glucose transporter and metabolism by membrane-bound hexokinase (1) and pentose phosphate-shunt enzymes (2) within macrophages

The Scheme indicates how ROI production is related to the pentose phosphate-shunt activity and how label is differentially lost from the C-1 and C-2,6 positions of 2dGlc.

be depleted relative to [C-2,6-³H]2dGlc. A simultaneous stimulation of both hexokinase activity and glucose-6-phosphate dehydrogenase activity should result in enhanced uptake of [C-2,6-³H]2dGlc above that seen with stimulation of hexokinase or glucose 6-phosphate activity alone. A stimulus to [C-1-³H]2dGlc will not necessarily be observed, as this may be relatively depleted owing to enhanced glucose-6-phosphate dehydrogenase activity.

The total amount of 2dGlc transported into the cell can be assessed by the rate of uptake of [C-2,6-³H]2dGlc; the rate of pentose phosphate-pathway activity can be assessed by the difference between the rates of [C-2,6-³H]2dGlc and [C-1-³H]2dGlc uptake, and the net accumulation of intracellular hexose phosphates and free 2dGlc can be assessed from the amount of [C-1-³H]2dGlc in the free sugar or hexose phosphate pool/10¹⁰ cells.

RESULTS

Effects of phorbol on 2dGlc uptake into rat thymocytes

There is no significant difference between C-1-³H- or C-2,6-³H-labelled 2dGlc uptake in either control or phorbol-treated thymocytes over the concentration range 0.01–1 mM (Table 1). Sugar with either label is accumulated to an intracellular concentration approx. by 8-fold in excess of the extracellular concentration (range 0.01–0.1 mM) in control, or by 14-fold in phorbol-treated

cells. At least 65 % of the intracellular sugar is in the free unphosphorylated state (Naftalin & Rist, 1989). Steady-state accumulation of free sugar within the cytosol above the concentration in the external solution can only result from coupling of sugar transport to hexokinase activity (Naftalin & Smith, 1987). The enhanced accumulation of free sugar in phorbol-treated cells indicates that the phorbol-dependent stimulus to 2dGlc uptake is related mainly to enhanced coupling between hexokinase activity and transport activity rather than to enhanced transport activity (Naftalin & Rist, 1990).

The equality of accumulation of [C-1-³H]- or [C-2,6-³H]-dGlc indicates that there is no significant pentose phosphate-pathway metabolism in rat thymocytes. These results show that, in the absence of a significant pentose phosphate pathway, 2dGlc, labelled either at the C-1 or the C-2,6 position is treated in the same way by both the transport and hexokinase reactions (Table 1).

Uptake of 2dGlc into rat and murine macrophages

As with rat thymocytes, there is evidence of an active accumulation process for 2dGlc into rat and murine macrophages. This accumulation process is linear over the first 15 min and saturable at raised concentrations of 2dGlc above 5 mM (R. J. Rist & R. J. Naftalin, unpublished work). There is an approx. 10-fold higher concentration of [C-1-³H]2dGlc in the cytosol

Table 1. Accumulation of 2dGlc into rat thymocytes

The accumulation of C-1-³H- and C-2,6-³H-labelled 2dGlc into rat thymocyte suspensions incubated in parallel in Hepes-buffered saline for 1 h containing 2dGlc at 0.01, 0.1 or 1.0 mM, with or without phorbol (40 nM) is shown. Phorbol ester significantly ($P < 0.01$) increased 2dGlc uptake at all concentrations. Results are presented as means $\pm$ S.E.M. Values in parentheses indicate the number of independent estimates.

[2dGlc] (mM)	Treatment ... Label ...	Accumulation (μ mol/60 min per 10 ¹⁰ cells)			
		Control		Phorbol	
		C-1	C-2,6	C-1	C-2,6
0.01		0.070 $\pm$ 0.005 (4)	0.08 $\pm$ 0.01 (4)	0.12 $\pm$ 0.01 (4)	0.14 $\pm$ 0.01 (4)
0.1		0.74 $\pm$ 0.03 (7)	0.74 $\pm$ 0.03 (7)	1.25 $\pm$ 0.08 (7)	1.34 $\pm$ 0.08 (7)
1.0		3.1 $\pm$ 0.3 (4)	3.1 $\pm$ 0.2 (4)	4.6 $\pm$ 0.3 (4)	4.6 $\pm$ 0.5 (4)

Table 2. Accumulation of 2dGlc into rat and murine macrophages

The accumulation of 2dGlc (0.1 mM, C-1 label only) into suspensions of rat or murine peritoneal macrophages during 3 h incubation in Hepes-buffered saline is shown. Free 2dGlc and 2dGlc6P are estimated by ion exchange fractionation, as described in the Methods section. Results are expressed as means $\pm$ S.E.M.; n is the number of independent estimates. Values marked $\dagger$ indicate that the free sugar concentration within the cytosol is raised significantly above the external sugar concentration ($\dagger\dagger P < 0.01$; $\dagger P < 0.05$). Those marked * indicate the values are significantly larger than the appropriate control values in the same species ($** P < 0.01$; $* P < 0.05$).

	Accumulation (μ mol/10 ¹⁰ cells)			
	Rat			Murine
	Control	mCSF-1	Phorbol	Control
Total	1.97 $\pm$ 0.04	4.40 $\pm$ 0.02**	2.00 $\pm$ 0.04	0.81 $\pm$ 0.05
Free sugar	1.31 $\pm$ 0.11 $\dagger\dagger$	2.98 $\pm$ 0.10 $\dagger\dagger$ **	1.06 $\pm$ 0.06 $\dagger$	0.53 $\pm$ 0.04 $\dagger$
HexP	0.52 $\pm$ 0.11	0.98 $\pm$ 0.13*	0.66 $\pm$ 0.06	0.21 $\pm$ 0.02
$n \dots$	4	4	2	4

[1.3 ± 0.1 ($n = 4$) mM] than in the external solution (0.1 mM) (Table 2). This accumulation of free sugar is doubled by mCSF-1 [2.98 ± 0.1 mM ($n = 4$); two separate experiments; $P < 0.01$].

Phorbol (40 nM) does not increase the steady-state accumulation of free 2dGlc into rat macrophages (Table 2) above control levels, although it does increase the rate of uptake of 2dGlc (from 0.83 ± 0.05 $\mu\text{mol}/15$ min per 10^{10} cells (control) to 1.54 ± 0.08 $\mu\text{mol}/15$ min per 10^{10} cells (phorbol), as determined by the uptake of C-2,6- ^3H -labelled 2dGlc (Table 3). This disparity between steady-state accumulation of free sugar and the rate of uptake can be ascribed to loss of [C-1- ^3H]2dGlc via the pentose phosphate pathway in phorbol-treated rat macrophages.

Effects of phorbol on 2dGlc uptake into murine macrophages

Uptake of 2dGlc into murine macrophages is less than 25% of the rate observed in rat macrophages (Tables 3 and 4). Phorbol stimulates 2dGlc uptake into mouse macrophages from 0.30 ± 0.02 to 0.51 ± 0.03 $\mu\text{mol}/30$ min per 10^{10} cells ($P < 0.01$). Phorbol stimulates [C-1- ^3H]- and [C-2,6- ^3H]2dGlc uptake equally into murine macrophages, indicating that there is no significant pentose phosphate-pathway activity in murine macrophages stimulated by phorbol alone. This contrasts with the effect of phorbol on rat macrophages.

Effects of mCSF-1 on 2dGlc uptake and accumulation in rat and mouse macrophages

Preincubation of rat macrophages for 1 h in buffered Hepes/saline containing mCSF-1 (1000 units/ml) increases the rate of accumulation of both [C-1- ^3H]- and [C-2,6- ^3H]2dGlc (Table 3) and also the steady-state accumulation of free 2dGlc (Table 2). mCSF-1 had a similar effect on the rate of 2dGlc uptake into mouse

Table 3. Uptake of 0.1 mM-2 dGlc into rat peritoneal macrophages

A comparison was made of the rates of uptake of C-1- and C-2,6-labelled 2dGlc (0.1 mM) into suspensions of rat peritoneal macrophages during a 15 min period immediately after preincubation for 1 h with or without mCSF-1 (1000 units/ml). Phorbol (40 nM) was added after the preincubation period. The results are expressed as means $\pm$ S.E.M. (n). Values marked * signify that the means are greater than the appropriate control ($P < 0.01$). Values marked † signify that the means for C-2,6-labelled 2dGlc uptake are greater than for C-1-labelled 2dGlc uptake ($P < 0.01$), and values marked ‡ signify that the uptake of C-2,6-labelled 2dGlc into cells incubated with both mCSF-1 and phorbol is greater than into cells incubated with either phorbol or mCSF-1 alone ($P < 0.01$).

Treatment	Label position	Uptake ($\mu\text{mol}/15$ min per 10^{10} cells)
Control	C-1	0.83 ± 0.05 (7)
	C-2,6	0.83 ± 0.05 (7)
Phorbol	C-1	0.81 ± 0.05 (6)
	C-2,6	1.54 ± 0.08 (8)*†
mCSF-1	C-1	1.27 ± 0.07 (6)*
	C-2,6	1.31 ± 0.05 (6)*n.s.
mCSF-1 + phorbol	C-1	0.85 ± 0.04 (6)
	C-2,6	2.09 ± 0.19 (6)*†‡

Table 4. Uptake of 2dGlc into murine macrophages

A comparison was made of the rates of uptake of C-1- and C-2,6-labelled 2dGlc (0.1 mM) into suspensions of murine peritoneal macrophages during a 30 min period immediately after preincubation for 1 h with or without mCSF-1 (1000 units/ml). Phorbol (40 nM), where present, was added after the preincubation period. The results are expressed as means $\pm$ S.E.M. (n). Values marked * are significantly larger than the appropriate control value; **, * $P < 0.01$ and < 0.05 respectively; values marked † are larger than the appropriate value with either phorbol or mCSF-1 alone; ††, † $P < 0.01$ or < 0.05 ; values marked with the symbol ‡ have a higher rate of uptake of C-2,6-labelled 2dGlc than of C-1-labelled 2dGlc under the same conditions ($P < 0.05$).

Treatment	Label position	Uptake ($\mu\text{mol}/30$ min per 10^{10} cells)
Control	C-1	0.30 ± 0.02 (8)
	C-2,6	0.31 ± 0.03 (8)
Phorbol	C-1	0.51 ± 0.03 (8)**
	C-2,6	0.51 ± 0.04 (8)**
mCSF-1	C-1	0.41 ± 0.02 (4)*
	C-2,6	0.44 ± 0.03 (4)*
mCSF-1 + phorbol	C-1	0.57 ± 0.04 (4)**†
	C-2,6	0.71 ± 0.05 (4)**†‡

macrophages (Table 4). These findings indicate an increase in hexokinase activity in cells exposed to CSF-1 without any concurrent stimulation of pentose phosphate-pathway metabolism.

Combined effects of mCSF-1 and phorbol on 2dGlc uptake and metabolism by macrophages

mCSF-1 and phorbol have a synergistic effect on 2dGlc uptake, as is evident from the enhanced uptake of C-2,6- ^3H -labelled 2dGlc above that seen in control suspensions, or in suspensions treated with either phorbol or mCSF-1 alone. This effect is observed in both rat and mouse macrophages, although the effect is larger in rat macrophages. It is evident from the enhanced difference between uptake of C-2,6- and C-1-labelled 2dGlc in both rat and mouse macrophages that a large component of the stimulus to sugar transport is due to an increase in pentose phosphate-pathway activity. With rat macrophages uptake of C-1-labelled 2dGlc in the presence of both mCSF-1 and phorbol is not different from the control (Table 3). However, in mouse macrophages the combination of mCSF-1 and phorbol enhances C-1-labelled-2dGlc uptake by a significant amount above the control (Table 4) and pentose phosphate-pathway activity is observed.

Effects of preloading with 1 mM-2dGlc on the uptake of labelled 2dGlc (0.1 mM)

The reason for investigating the effect of preloading with unlabelled 2dGlc, thereby generating a high intracellular concentration of both unlabelled free sugar and hexose phosphate within the cells (approx. 3 mM free sugar and 3 mM-hexose phosphate) is to ascertain whether this intracellular solute retards the rate of labelled 2dGlc (0.1 mM) uptake into the cells. A significant reduction in uptake would signify that the labelled sugar enters the whole-cell pool of sugar metabolites and

Table 5. Effects of preloading with 1 mM unlabelled 2dGlc on uptake of C-1-³H- and C-2,6-³H-labelled 2dGlc (0.1 mM) into rat macrophages

Rat macrophages were preincubated for 1 h in Hepes-buffered saline with or without unlabelled 1 mM-2-dGlc and also with or without phorbol (40 nM). After preincubation the cells were transferred to fresh medium containing C-1- or C-2,6-labelled 2dGlc (0.1 mM) with or without phorbol (40 nM) and incubated for a further 1 h. The results are expressed as means $\pm$ S.E.M.; *n* is number of independent estimates. Values marked * indicate C-2,6-labelled 2dGlc uptake into phorbol-treated cells significantly higher than into either C-1-labelled cells with or without phorbol and C-2,6-labelled control cells (*P* < 0.01). The value marked † shows a significantly lower rate of C-1-labelled 2dGlc uptake into 2dGlc-preloaded cells than into cells which have been preincubated in sugar-free solutions.

Treatment	Preload (mM unlabelled 2dGlc)	Label position	Uptake (μ mol/60 min per 10 ¹⁰ cells)
Control	0	C-1	0.89 $\pm$ 0.02 (5)
		C-2,6	0.98 $\pm$ 0.04 (5)
Phorbol	1	C-1	0.88 $\pm$ 0.08 (5)
		C-2,6	0.93 $\pm$ 0.09 (5)
	0	C-1	0.92 $\pm$ 0.04 (5)
		C-2,6	1.98 $\pm$ 0.05 (5)*
	1	C-1	0.70 $\pm$ 0.02 (5)†
		C-2,6	1.92 $\pm$ 0.04 (5)*

its specific radioactivity consequently reduced before incorporation into either the hexose phosphate pool or the pentose phosphate pathway. Preloading with 1 mM-2dGlc has no significant effect on uptake of C-2,6 label in either control or phorbol-stimulated states (Table 5), although it does cause a small reduction in incorporation of C-1 label into phorbol-treated cells. These results indicate that 2dGlc is transformed to either 2dGlc6P or 2-deoxyribose 5-phosphate before equilibrating with the cell pools of these metabolites.

DISCUSSION

The results indicate that control of 2dGlc accumulation in macrophages is exerted via a dual system. mCSF-1 enhances the hexokinase-coupled uptake of 2dGlc into rat and mouse macrophages, as is evident from the enhanced rate of accumulation of the free sugar and the absence of any difference in the rate of accumulation of uptake of C-1- or C-2,6-labelled 2dGlc into either rat or murine macrophages (Tables 2, 3 and 4). These data confirm the findings of Hamilton *et al.* (1988) and additionally show that the stimulus to rat macrophage glucose transport by mCSF-1 is not identical with that exerted by phorbol. The results also show that stimulation of 2dGlc transport alone is not sufficient to activate the pentose phosphate pathway. Thus this result indicates that induction of pentose phosphate-shunt activity and ROI production necessitates activation of some additional step to that required for activation of sugar transport and accumulation.

Phorbol stimulates 2dGlc uptake in rat macrophages to the same extent as does CSF-1. The phorbol-dependent

stimulus in the rate of uptake of 2dGlc into rat macrophages is apparently due to increased pentose phosphate-pathway activity. However, the phorbol-dependent enhancement of labelled uptake of 2dGlc via the pentose phosphate-pathway activity requires, among other things, an increased rate of production of 2dGlc6P via hexokinase (Scheme 1). Part of the increased hexokinase activity may be due to a reduced product inhibition resulting from increased utilization of 2dGlc6P via the pentose phosphate pathway. Evidence for this comes from the relative decrease in accumulation of [C-1-³H]2dGlc in the phorbol-treated suspensions compared with the amount of [C-2,6-³H]2dGlc found in phorbol-treated cells and also in comparison with the amount of [C-1-³H]2dGlc found in mCSF-1-treated cells (Table 3). However, the data could also be consistent with a small increase in endofacial hexokinase activity due to additional enzyme being present, together with a large increase in pentose phosphate-pathway activity. The reaction of murine macrophages to phorbol differs from that of rat macrophages in that there is stimulation of 2dGlc without any differential uptake of the C-1 or C-2,6 label. Hence phorbol (40 nM) by itself does not appear to stimulate murine macrophage pentose phosphate-pathway activity.

A possible explanation for the synergistic increase in 2dGlc uptake into rat macrophages induced by the combined effect of CSF-1 and phorbol is that the stimulus to the pentose phosphate pathway seen with phorbol alone is no longer rate-limited by low hexokinase activity at the endofacial surface of the transporter, as this is stimulated independently after ligand binding to CSF-1 receptors. Whether these separate controls of glucose metabolism at the membrane surface affect the long-term synergistic effect on macrophage development is uncertain and requires further investigation.

The mCSF-1-dependent stimulus to sugar transport in isolation from effects on pentose phosphate-pathway activity may provide a previously unsuspected additional pathway through which CSF-1 may sustain macrophages in culture (Walker *et al.*, 1985).

The absence of any substantial effect of preloading the cells with unlabelled sugar on the rates of uptake on [C-1-³H]- or [C-2,6-³H]-dGlc (Table 5) indicates that the newly emergent sugar bound to the endofacial surface of the transporter is preferentially utilized by hexokinase and the pentose phosphate pathway rather than the substrate already present within the cytosol. This finding indicates that the metabolites are 'tunnelled' within a complex of enzymes bound to the membrane surface, with very little leakage between the cytosolic pool and the surface-bound metabolites (see Scheme 1). This may mean that cytosolic stores are conserved during the energy-dissipating activity involved in production of ROIs. A possible practical consequence of this view is that the rate of pentose phosphate-shunt activity, and hence of the cytotoxic ROI production, could be rapidly controlled by agents which inhibit sugar transport and or hexokinase activity.

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Received 24 May 1989/11 August 1989; accepted 18 August 1989