Acute regulation of glucose transport in a monocyte–macrophage cell line: Glut-3 affinity for glucose is enhanced during the respiratory burst

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Activation of the respiratory burst imposes acute metabolic demands on phagocytic cells. These are met by mobilizing internal energy stores and by increasing the utilization of exogenous energy, including glucose in the circulation. To determine whether the increased glucose uptake that is known to be associated with the respiratory burst involves the regulation of glucose transporter molecules, the intrinsic transport properties of glucose transporters on the macrophage cell line RAW 264.7 were determined after activation with PMA, *N*-formyl-methionine-leucine-phenylalanine (fMLP) and the cytokines granulocyte/macrophage colony-stimulating factor (GM-CSF) and interleukin 3 (IL-3). Treatment with PMA resulted in a 2-fold increase in respiratory burst activity within 10 min; this was associated with a 30–50 % increase in 2-deoxyglucose uptake and a 4-fold increase in transporter affinity for glucose. Similarly, fMLP, GM-CSF and IL-3 treatments stimulated 2-deoxyglucose uptake that was associated with a 3–4-fold increase in transporter affinity for glucose. To determine whether the changes observed in 2-deoxyglucose uptake in response to PMA, fMLP and growth factors were influenced by phosphorylation of the sugar, 3-*O*-methylglucose, which is not phosphorylated, was used. Increased 3-*O*-methylglucose uptake and increased transporter affinity for glucose were also observed after PMA, fMLP and GM-CSF treatments. Whereas both fMLP and GM-CSF stimulated superoxide production, IL-3 failed to activate respiratory burst activity. The protein kinase inhibitors genistein and staurosporine inhibited the increase in 2-deoxyglucose uptake observed with fMLP and GM-CSF, and partly reversed the affinity increase towards that of untreated control cells. In contrast, the phosphatidylinositol 3-kinase inhibitor wortmannin had little effect on 2-deoxyglucose uptake in response to these activators. Western blotting with subtype-specific antisera showed that Glut-3 was the predominant transporter on RAW 264.7 cells. These studies demonstrate that acute regulation of glucose transporters occurs in response to activators that promote respiratory burst activity, and show that this regulation involves both tyrosine kinases and protein kinase C activity.

INTRODUCTION

Activation of macrophages and neutrophils by pathogens and non-physiological stimuli produces a respiratory burst in which oxygen consumption increases markedly [1]. Oxygen is utilized by membrane-bound NADPH oxidase to generate superoxide radicals, which contribute to the destruction of invading micro-organisms. With macrophages, the energy needed to fuel NADPH oxidase derives from extracellular glucose that is metabolized via the hexose monophosphate shunt pathway to produce NADPH [2,3]. In contrast, neutrophils are capable of mobilizing stored glycogen to fuel the respiratory burst in addition to utilizing exogenous glucose.

Transport of glucose across the plasma membrane occurs in most cells via a family of structurally related 'facilitative' glucose transporter molecules that shift glucose down its concentration gradient without expenditure of energy [4]. These transporters are often expressed in a tissue-specific or cell-specific manner and in some cases their expression is regulated by extracellular signals such as hormones and growth factors [5]. In adipose and muscle cells, in the short term, increased glucose transport in response to insulin occurs by translocation of Glut-4 from an intracellular pool to the plasma membrane [6,7]. Glut-1 is also recruited to the plasma membrane in response to insulin, although this occurs to a much smaller extent than for Glut-4. In addition to increased plasma membrane expression, there is also evidence that the intrinsic activities of Glut-1 and Glut-4 are modulated in response to insulin [8–11].

Although an adequate supply of glucose is crucial for basal cell metabolism and thus for maintaining cell viability, the regulation of glucose transport into cells in systems other than those involving insulin is poorly understood. Stress induced by a variety of reagents stimulates glucose transport by translocating transporters from intracellular sites to the plasma membrane [12] or by increasing transporter expression [13]. In contrast, glucose deprivation or Cd^{2+} treatment of 3T3L1 cells [14,15] or treatment of Chinese hamster V29 lung fibroblasts with allose [16] modulates the intrinsic activity of Glut-1. Furthermore treatment of rat liver clone 9 cells with azide promotes glucose transport without increasing Glut-1 expression at the plasma membrane or transporter affinity for glucose [17,18]. The mechanism of the increased glucose transport in this system remains unclear. In haemopoietic cells, we and others have shown that interleukin 3 (IL-3) and other growth factors stimulate glucose transport in growth-factor-dependent cells by increasing the affinity of glucose transporters for glucose without a change in transporter expression or V_{max} [19,20]. In other studies, the malignant phenotype and the presence of acutely transforming viruses and oncogenes

Abbreviations used: Con A, concanavalin A; 2-DOG, 2-deoxy-D-glucose; fMLP, *N*-formyl-methionine-leucine-phenylalanine (chemotactic peptide); GM-CSF, granulocyte/macrophage colony-stimulating factor; IL-3, interleukin 3; 3-*O*-MG, 3-*O*-methylglucose; PKC, protein kinase C; PI 3-kinase, phosphatidylinositol 3-kinase; TBS/T, Tris-buffered saline/0.05 % Tween-20.

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have been associated with increased transporter affinity for glucose [21], whereas apoptosis induced in human Jurkat cells by an antibody against CD95 markedly decreased the affinity of Glut-1 for glucose [22].

Haemopoietic growth factors such as CSF-1 and granulocyte/macrophage colony-stimulating factor (GM-CSF), and activating molecules such as PMA, enhance glucose uptake in bone-marrow-derived macrophages, resident peritoneal macrophages and macrophage cell lines of murine origin [2,3,23]. With rat peritoneal macrophages stimulated with CSF-1 and PMA, increased 2-deoxy-D-glucose (2-DOG) uptake was associated with a 39% increase in transporter affinity for glucose [3]; this was correlated with hexokinase translocation to the plasma membrane and coupling to sugar transport [24]. With CSF-1, increased glucose transport was dissociated from superoxide production and effects on the hexose monophosphate shunt. In contrast, with PMA, increased glucose transport was dependent on both superoxide production and stimulation of the hexose monophosphate shunt [3]. With the murine macrophage cell line J774.16, PMA-stimulated 2-DOG uptake was not associated with a change in affinity of the transporter for glucose but with increased V_{max} [2]. Although early studies with human peripheral blood neutrophils failed to link the respiratory burst and superoxide production with increased 2-DOG uptake after treatment with PMA [25,26], current studies in our laboratory have provided contradictory results (A. S. Tan and M. V. Berridge, unpublished work). Comparison of the methodologies shows that neutrophils isolated by plasma gel methods and hypotonic lysis transport glucose poorly and show characteristics of activated cells.

In the present study we investigated the acute regulation of glucose transport that is associated with respiratory burst activation in the murine macrophage cell line RAW 264.7. At optimum times of exposure to PMA, *N*-formyl-methionine-leucine-phenylalanine (fMLP) and GM-CSF, increased superoxide production was associated with increased 2-DOG and 3-*O*-methylglucose (3-*O*-MG) uptake and increased transporter affinity for glucose. The protein kinase inhibitors genistein and staurosporine inhibited the increased 2-DOG uptake observed, and partly reversed the increased affinity associated with cell activation. Glut-3 was shown to be the major transporter expressed on RAW 264.7 cells. These results demonstrate that the activation of a murine macrophage cell line involves an acute change in the intrinsic activation state of Glut-3.

MATERIALS AND METHODS

Cells and cell culture

The mouse macrophage cell line RAW 264.7 was obtained from the American Type Culture Collection. Cells were maintained in RPMI 1640 medium (Gibco-BRL, Grand Island, NY, U.S.A.) supplemented with 25 μg/ml penicillin, 25 μg/ml streptomycin and 10% (v/v) fetal calf serum. Recombinant murine IL-3 and GM-CSF were obtained from Professor J. D. Watson (Genesis Research and Development Corporation, Auckland, New Zealand). Cells were maintained at 37 °C in a humidified incubator maintained in air/CO_2 (19:1, v/v). Cell viability was determined by Trypan Blue exclusion using a haemocytometer.

Chemicals

Calphostin C was obtained from Kamiya Biomedical Company (Thousand Oaks, CA, U.S.A.) and 2-DOG from Fluka (Buchs, Switzerland). All other chemicals including the stimulants and inhibitors were from Sigma Chemical Company (St. Louis, MO, U.S.A.).

[^{3}H]Thymidine incorporation assay

Proliferative responses were measured by incubating 5×10^4 cells in 0.1 ml of culture medium in 96-well microtitre plates for periods of 16–20 h before adding 0.5 μCi of [^{3}H]thymidine (Amersham, Little Chalfont, Bucks., U.K.) for 3 h. The incorporation of radioactivity into DNA was determined with an automated cell harvester and liquid-scintillation counting.

[^{3}H]2-DOG uptake

Glucose uptake was measured by the zero-trans method with [^{3}H]2-DOG as described previously [20]. Briefly, exponentially growing RAW cells were washed in RPMI 1640 with or without 10% (v/v) fetal bovine serum, resuspended at 5×10^5 cells/ml and allowed to adhere for 1 h at 37 °C. Cells were treated with or without growth factors and/or other stimulants in a total volume of 1.0 ml for the respective lengths of time. Cells were then washed twice by aspiration and preincubated at 37 °C for 5 min in glucose-free RPMI 1640. [^{3}H]2-DOG (200 μM, 0.5 μCi; Amersham) was then added and glucose uptake was determined for 5 min under conditions where the uptake was linear for 8 min. Uptake was stopped by the addition of 500 ml of ice-cold glucose-free RPMI 1640 containing 0.3 mM phloretin and chilling on ice for a further 5 min. Cells were solubilized in 100 ml of 1% (v/v) Triton X-100 and radioactivity was determined by liquid-scintillation counting. All kinetic analyses of 2-DOG uptake used 0.1–2.5 mM 2-DOG in the extracellular medium and were measured over 5 min. The effects of the growth factors PMA and fMLP on glucose uptake were determined by adding them to exponentially growing cells at 37 °C for the respective periods. Cells were washed free of the added factors; glucose transport was measured and kinetic analysis was carried out as described above. Where inhibitors were used, cells were treated with the inhibitors for 90 min before the addition of the growth factors PMA and fMLP.

[^{3}H]3-*O*-MG uptake

The uptake of 3-*O*-MG was determined by a similar procedure to that described above for 2-DOG uptake except that the reaction was performed at 20 °C and was terminated after 5 s, at which time uptake was in the linear range.

Cytochrome *c* reduction

The rate of production of superoxide was used as a measure of the oxidative burst and was evaluated by the discontinuous spectrophotometric assay of superoxide-inhibitable reduction of ferricytochrome *c* to the ferrous form. Briefly, cells (2×10^6) were incubated in 1 ml of Hanks balanced salt solution [5.4 mM KCl/0.3 mM Na_2HPO_4/0.4 mM KH_2PO_4/4.2 mM $NaHCO_3$/1.3 mM $CaCl_2$/0.5 mM $MgCl_2$/6 mM $MgSO_4$/137 mM NaCl/5.6 mM glucose (pH 7.4)] containing 120 μM cytochrome *c*. The reaction was initiated by adding PMA (1 μM), fMLP (1 μM) or GM-CSF (100 ng/ml) in the presence or absence of superoxide dismutase (90 units/ml). All incubations were performed at 37 °C for 10 min. The reaction was stopped by placing

the tubes on ice for 5 min, after which the cells were removed by centrifugation at 1200 **g** for 2 min. The supernatant containing reduced cytochrome *c* was measured at 550 nm against a blank containing all reagents except the cells.

Preparation of crude membranes

Cells were collected, washed in PBS and resuspended in Tris buffer [10 mM Tris/HCl (pH 7.4)/1 mM EDTA/200 mM sucrose/1 mM PMSF]. Resuspended cells were sonicated and cellular debris was removed by centrifugation at 900 **g** for 10 min at 4 °C. The supernatant was centrifuged at 110000 **g** for 75 min and the crude membrane pellet was solubilized in 10 mM Tris/HCl, pH 7.4 containing 0.5 % Triton X-100 and 1 mM PMSF for 1 h at 4 °C. A concentration of 0.5 % Triton X-100 in the solubilization buffer gave a maximum yield of glucose transporters. Insoluble material was removed by centrifugation for 5 min in a Microfuge and the solubilized membranes were stored at −70 °C.

Western blot analysis

Solubilized cell membrane preparations (15 μg) were added to Laemmli sample buffer and incubated at 37 °C for 10 min. Proteins were separated on SDS/8 % polyacrylamide gels with a Mini-Protean II apparatus (Bio-Rad Laboratories, Hercules, CA, U.S.A.). Proteins were transferred electrophoretically to supported nitrocellulose membranes (Hybond-C Super; Amersham) at 15 V for 30 min. Non-specific binding to membranes was blocked by incubating in Tris-buffered saline/0.05 % Tween-20 (TBS/T) containing 5 % (w/v) non-fat dried milk and 5 % (w/v) BSA for 1 h at room temperature. Membranes were then incubated with primary antisera diluted in TBS/T containing 1 % (w/v) BSA for 2 h at room temperature. Rabbit polyclonal antiserum against the C-terminus of rat Glut-1, Glut-4 (1:200 dilution; EastAcres Biologicals, Southbridge, MA, U.S.A.) and affinity-purified antibodies against the C-terminus of mouse Glut-3 [1:100 dilution, gift from G. W. Gould (University of Glasgow, Glasgow, U.K.)] were employed. Normal rabbit serum was used as a control in each case. Membranes were washed with TBS/T and then incubated for 1 h with affinity-purified pig Ig anti-(rabbit IgG) conjugated to alkaline phosphatase (Sigma) diluted 1:4000 in TBS/T. Membranes were washed and developed with 0.05 % Nitro Blue Tetrazolium and 0.01 % 5-bromo-4-chloro-3-indolyl phosphate in alkaline phosphatase buffer [100 mM Tris/HCl (pH 9.8)/100 mM NaCl/10 mM $MgCl_2$] for 8 min. The reaction was stopped by rinsing the membrane with distilled water.

Protein determination

Protein was determined with a microplate adaptation of the Bradford method [27]; the absorbance was measured at 570 nm.

RESULTS

Effects of PMA, fMLP, GM-CSF and IL-3 on superoxide production by RAW cells

RAW 264.7 cells were treated with PMA or fMLP or the growth factors GM-CSF or IL-3 for 10 min in the presence of ferricytochrome *c*, and superoxide production was determined as superoxide-inhibitable cytochrome *c* reduction. Superoxide production was measured at 5 mM glucose. Table 1 shows that superoxide production was stimulated by 73–92 % by PMA, fMLP and GM-CSF compared with untreated cells. No significant response to IL-3 was observed. In contrast, proliferative responses at 16 h were inhibited by 35–45 % by PMA and fMLP but remained unaffected by GM-CSF and IL-3.

Effects of PMA, fMLP, GM-CSF and IL-3 on uptake of [^{3}H]2-DOG by RAW cells

To determine whether the respiratory burst of RAW cells was associated with increased uptake of 2-DOG, cells were treated with PMA, fMLP or concanavalin A (ConA), or with GM-CSF or IL-3 in the presence or absence of serum for periods of up to 1 h before measurement of [^{3}H]2-DOG uptake over 5 min. PMA-stimulated 2-DOG uptake by RAW cells was independent of serum. In contrast, responses to fMLP were increased markedly in the presence of serum; Con A responses were also serum-dependent. With GM-CSF and IL-3, serum inhibited the stimulation of 2-DOG uptake by growth factors (results not shown). Figure 1 (left panel) shows that in the presence of serum, fMLP stimulated 2-DOG uptake by a maximum of 160 % at 15 min and that the response declined rapidly thereafter. With PMA and Con A, peak responses of 75 % and 150 % were observed at 30 min, whereas with IL-3 and GM-CSF (Figure 1, right panel), cellular responses determined in the absence of serum peaked at 30–60 min. Table 2 shows that 2-DOG uptake enhanced by PMA, fMLP, GM-CSF and IL-3 was associated with a 3–4-fold increase in transporter affinity for glucose. No significant changes in V_{max} were observed after IL-3 and GM-CSF treatment; however, V_{max} declined with PMA.

Effects of PMA, fMLP and GM-CSF on uptake of [^{3}H]3-*O*-MG by RAW cells

To determine whether post-transport phosphorylation of 2-DOG affected uptake and activation by PMA, fMLP and growth factors, the kinetics of uptake of 3-*O*-MG, a glucose analogue that cannot be phosphorylated, was determined. A major problem in using 3-*O*-MG to measure transport was that uptake was very rapid and was linear for less than 10 s. Thus uptake was measured at 20 °C over 5 s.

Table 3 shows that PMA and fMLP stimulated 3-*O*-MG uptake by 30–50 %, whereas GM-CSF enhanced uptake 2.5-fold. Stimulation of 3-*O*-MG uptake in response to PMA and fMLP was associated with a 30 % decrease in K_m compared with untreated cells, whereas GM-CSF treatment halved the K_m. These results show a similar trend to those observed with 2-DOG, although quantitative differences are evident.

Effect of protein kinase inhibitors and wortmannin on 2-DOG uptake by RAW cells

To determine whether increased 2-DOG uptake in response to PMA, fMLP and growth factors was associated with protein kinase activity, cells were treated with genistein (a tyrosine kinase inhibitor), staurosporine and calphostin C [specific protein kinase C (PKC) inhibitors] and wortmannin [a phosphatidylinositol 3-kinase (PI 3-kinase) inhibitor] for 1 h before adding PMA, fMLP or GM-CSF. Table 4 shows that genistein, staurosporine and calphostin C inhibited the 2-DOG uptake stimulated by fMLP and GM-CSF, but had much smaller effects on basal 2-DOG uptake. Under the same experimental conditions, wortmannin at concentrations up to 500 nM had little effect on 2-DOG uptake. To determine whether the inhibitory effects of genistein and staurosporine on fMLP- and GM-CSF-stimulated 2-DOG uptake involved changes in the affinities of the transporters for glucose, kinetic analysis was performed in the presence of these

Table 1 Effects of PMA, fMLP, GM-CSF and IL-3 on [^{3}H]thymidine incorporation and respiratory burst activity by RAW cells

Cells grown in RPMI 1640 were treated with PMA (1 μM), fMLP (1 μM), GM-CSF (100 ng/ml) or IL-3 (20 ng/ml) for 16 h before adding [^{3}H]thymidine for 3 h. Respiratory burst activity was determined by stimulating the cells for 10 min in Hanks balanced salt solution and measuring superoxide-inhibitable reduction of ferricytochrome *c* at 550 nm. Values are means ± S.E.M. for a minimum of three separate experiments performed in triplicate. *$P < 0.02$ compared with control cells.

Cell treatment	[^{3}H]Thymidine incorporation (c.p.m. per 5×10^4 cells)	[^{3}H]Thymidine incorporation (% of control)	Superoxide production (nmol/min per 10^6 cells)	Superoxide production (% of control)
Control	5268 ± 224	100 ± 5	10.75 ± 0.7	100 ± 6
PMA	3440 ± 157*	65 ± 4*	20.65 ± 0.7*	192 ± 3*
fMLP	2897 ± 88*	55 ± 3*	19.68 ± 0.6*	183 ± 3*
GM-CSF	5433 ± 360	103 ± 6	18.60 ± 0.6*	173 ± 3*
IL-3	5347 ± 523	102 ± 7	9.97 ± 0.7	92 ± 6

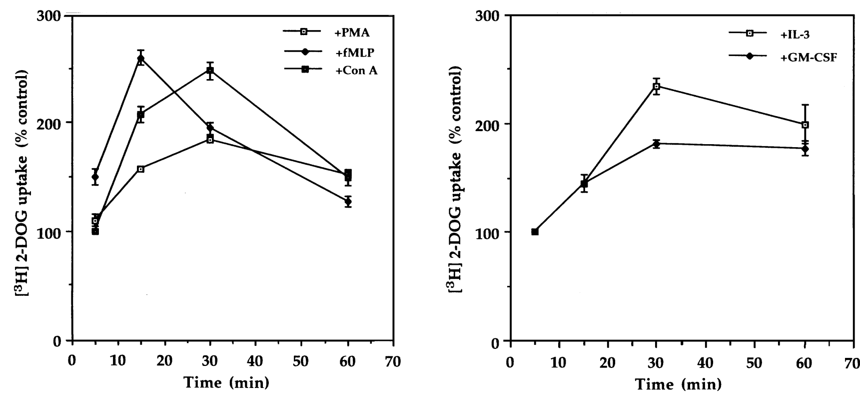

Figure 1 Effects of PMA, fMLP, Con A, IL-3 and GM-CSF on 2-DOG uptake by RAW cells

RAW cells were stimulated with (left panel) PMA (1 μM), fMLP (1 μM) or Con A (10 μg/ml) in the presence of 10% (v/v) serum or (right panel) GM-CSF (100 ng/ml) or IL-3 (50 ng/ml) in the absence of serum for various times, and uptake of [^{3}H]2-DOG was determined. Values are means ± S.E.M. for a minimum of three separate experiments performed in triplicate.

Table 2 Effect of growth factors and other stimulants on [^{3}H]2-DOG transport kinetics by murine RAW cells

Cells were preincubated in RPMI 1640 at 37 °C for 10 min before stimulation with PMA (1 μM), fMLP (1 μM), GM-CSF (100 ng/ml) or IL-3 (20 ng/ml). [^{3}H]2-DOG uptake at increasing 2-DOG concentrations (0.1–2.5 mM) was analysed by Lineweaver–Burk plots. Values are means ± S.E.M. for a minimum of four separate experiments involving triplicate determinations. *$P < 0.05$, **$P < 0.01$ compared with control cells.

Cell treatment	[^{3}H]2-DOG uptake (nmol/min per 10^6 cells)	K_m (mM)	V_{max} (nmol/min per 10^6 cells)
Control	0.09 ± 0.01	6.13 ± 2.40	2.88 ± 0.60
PMA (30 min)	0.15 ± 0.01*	2.03 ± 0.62*	1.45 ± 0.28
fMLP (15 min)	0.34 ± 0.14**	1.37 ± 0.29*	2.37 ± 0.60
GM-CSF (60 min)	0.34 ± 0.05**	1.22 ± 0.05*	2.80 ± 0.90
IL-3 (60 min)	0.41 ± 0.14**	1.54 ± 0.50*	3.00 ± 0.22

Table 3 Effect of growth factors and other stimulants on [^{3}H]3-*O*-MG transport kinetics by murine RAW cells

Cells were stimulated with PMA (1 μM), fMLP (1 μM) or GM-CSF (100 ng/ml) before measurement of 3-*O*-MG uptake. [^{3}H]3-*O*-MG uptake at increasing 3-*O*-MG concentrations (0.1–2.5 mM) was performed at 20 °C and data were analysed by Lineweaver–Burk plots. Values are means of two separate experiments involving triplicate determinations. Variation between experiments was 10–20%.

Cell treatment	[^{3}H]3-*O*-MG uptake (nmol/s per 10^6 cells)	K_m (mM)	V_{max} (nmol/s per 10^6 cells)
Control	0.17	10	9.56
PMA (30 min)	0.26	7.32	8.12
fMLP (15 min)	0.22	7.14	6.58
GM-CSF (60 min)	0.52	5.50	14.70

Table 4 Effect of inhibitors of PKC, tyrosine kinases and PI 3-kinase on fMLP- and GM-CSF-stimulated [^{3}H]2-DOG uptake

Cells were treated with genistein (500 μM), calphostin C (50 nM), staurosporine (4 μM) or wortmannin (500 nM) for 90 min before stimulation with fMLP (1 μM) or GM-CSF (100 ng/ml) for 30, 15 or 60 min respectively. Values are means ± S.E.M. for three separate experiments, each performed in triplicate. ** $P < 0.01$ compared with control; †† $P < 0.01$ compared with stimulated cells.

Cell treatment	[^{3}H]2-DOG uptake (nmol/min per 10^6 cells)	[^{3}H]2-DOG uptake (% of control)	Inhibition of stimulation (%)
Control	0.09 ± 0.01	100 ± 5	–
+ genistein	0.07 ± 0.00	80 ± 2	–
+ staurosporine	0.07 ± 0.00	80 ± 4	–
+ calphostin C	0.07 ± 0.01	70 ± 6	–
+ wortmannin	0.08 ± 0.01	88 ± 5	–
fMLP	0.34 ± 0.14	377 ± 40**	–
+ genistein	0.17 ± 0.01	188 ± 6††	50 ± 1.6
+ staurosporine	0.21 ± 0.03	233 ± 14††	40 ± 1.3
+ calphostin C	0.22 ± 0.02	244 ± 9††	35 ± 1.3
+ wortmannin	0.30 ± 0.01	333 ± 3**	12 ± 0.1
GM-CSF	0.34 ± 0.05	377 ± 14**	–
+ genistein	0.19 ± 0.00	211 ± 5††	45 ± 1.2
+ staurosporine	0.22 ± 0.02	244 ± 9††	36 ± 1.3
+ calphostin C	0.22 ± 0.04	244 ± 18††	36 ± 2.6
+ wortmannin	0.32 ± 0.01	355 ± 3**	6 ± 0.05

Table 5 Effect of inhibitors of PKC and tyrosine kinases on fMLP- and GM-CSF-stimulated [^{3}H]2-DOG uptake kinetics

Cells were treated with genistein (500 μM) or staurosporine (4 μM) for 90 min before stimulation with fMLP (1 μM) or GM-CSF (100 ng/ml) for 30, 15 or 60 min respectively. Values are means ± S.E.M. for a minimum of three separate experiments, each performed in triplicate.

Cell treatment	K_m (mM)	V_{max} (nmol/min per 10^6 cells)
Control*	6.13 ± 2.40	2.88 ± 0.60
fMLP*	1.37 ± 0.29	2.37 ± 0.60
fMLP + genistein	2.35 ± 0.10	2.12 ± 0.07
fMLP + staurosporine	2.86 ± 0.32	3.20 ± 0.78
GM-CSF*	1.22 ± 0.05	2.80 ± 0.90
GM-CSF + genistein	3.12 ± 0.24	3.36 ± 0.22
GM-CSF + staurosporine	2.46 ± 0.48	3.27 ± 0.09

* Data from Table 2 repeated for comparison.

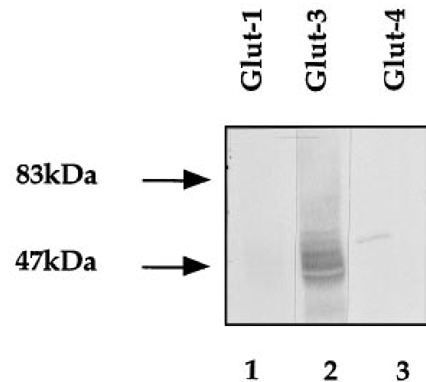

Figure 2 Expression of Glut-1, Glut-3 and Glut-4 in RAW cells

Crude membranes were prepared from cells grown at 37 °C and the transporter subtypes were analysed by Western blotting and alkaline phosphatase staining. Lane 1, Glut-1; lane 2, Glut-3; lane 3, Glut-4. Each lane contained 15 μg of protein.

inhibitors. Table 5 shows that with fMLP and GM-CSF, genistein and staurosporine partly reversed the increase in affinity that was associated with cell activation. A similar inhibition and affinity reversal was observed after staurosporine treatment of PMA-induced cells; however, with genistein, Lineweaver–Burk plots gave a negative slope, suggesting a confounding interaction between PMA and genistein. Genistein and staurosporine had little effect on the V_{max} of fMLP- and GM-CSF-stimulated cells.

Glut-3 is the major glucose transporter subtype on RAW cells

Although Glut-1 is often regarded as the universal glucose transporter present on most cells, including haemopoietic cells, there is evidence to suggest that other transporters might also contribute to glucose uptake by haemopoietic cells [28–30]. Using subtype-specific antisera against Glut-1, Glut-3 and Glut-4, we determined the transporter subtypes expressed on RAW cell membranes by Western blotting. Figure 2 shows that Glut-3 was strongly expressed on RAW cells, whereas Glut-1 and Glut-4 were not detected. In other experiments (M. Kansara, unpublished work) it has been demonstrated that Glut-3 is also the predominant glucose transporter subtype on murine peripheral blood mononuclear cells, whereas Glut-1 is predominant on the murine erythroleukaemia cell line 416B and on myelomonocytic WEHI 3B cells.

DISCUSSION

Increasing evidence suggests that extracellular signals regulate the transport activity of glucose transporter molecules at the plasma membrane. Thus changes in the kinetic properties of transporters, or changes in their specific activities, have been demonstrated in a variety of situations [3,8–11,14–22,31]. Although many of these studies have involved cells in which the glucose transporter subtypes have not been defined or in which a mixture of transporter subtypes has been present, other studies have provided clear evidence that the activity of a particular transporter changes [16–18,22,31].

In this study we describe the acute regulation of Glut-3 in a murine macrophage cell line, RAW 264.7, by activating molecules

that include PMA and fMLP and the haemopoietic growth factors GM-CSF and IL-3. Although Rist et al. [3] have previously demonstrated that the affinity of glucose transporters for glucose on rat peritoneal macrophages increases in response to respiratory burst activators such as PMA and CSF-1, the change in affinity observed was relatively small and not readily accessible to detailed analysis of the mechanism involved, and the transporter isotype was not determined. In the present study we have explored the regulation of glucose transport in a mouse macrophage cell line that expresses primarily Glut-3, a transporter not previously investigated with regard to acute regulation. The affinity of Glut-3 for glucose increased with activation. With fMLP, stimulation of 2-DOG uptake was rapid, a significant increase being observed within 5 min and maximum stimulation by 15 min. With PMA, Con A and the growth factors GM-CSF and IL-3, the effect on 2-DOG uptake was slower, maximum effects being observed at 30–60 min. These results differ from those of Kiyotaki et al. [2], who failed to detect a change in affinity for 2-DOG after treatment of J774.16 cells with PMA, but rather observed a 2-fold increase in V_{max}. In J774.16 cells and in a variant clone with a defect in oxidative metabolism, K_m values for 2-DOG uptake of approx. 1 mM were reported in both unstimulated and PMA-treated cells. This is about the same affinity observed in RAW 264.7 cells stimulated with PMA, fMLP, GM-CSF and IL-3. If the same transporter isotype is involved with both cell lines, these results could be explained by constitutive activation of the glucose transporter on J774.16 cells. In contrast, both RAW 264.7 cells and rat peritoneal macrophages seem to have the capacity for affinity regulation despite the fact that different levels of affinity switching are involved.

Uptake of 2-DOG is a coupled reaction in which the transport of hexose is linked to its subsequent phosphorylation. In contrast, 3-*O*-MG is not phosphorylated by hexokinase and the uptake measured is independent of sugar phosphorylation. The uptake and kinetics of 3-*O*-MG transport showed similar trends to those obtained with 2-DOG, indicating a clear relationship between the enhancement of glucose transport and the increase in glucose transporter affinity in response to respiratory burst activation by PMA, fMLP and GM-CSF. Although the changes in K_m values in response to PMA and fMLP are not as pronounced as those observed with 2-DOG, increased uptake is again associated with increased affinity for 3-*O*-MG. Hence the differences between 2-DOG and 3-*O*-MG uptake and K_m could be explained by the phosphorylation of 2-DOG by hexokinase. Higher K_m values were observed when 3-*O*-MG was used; this has been reported by other investigators using other cell types [32–36]. Differences in uptake between the two hexoses were also observed and these would have contributed to the differences in V_{max}. Direct comparisons of uptake and kinetics between the two hexoses should be viewed with caution because of the different experimental conditions used and limited linearity of 3-*O*-MG uptake.

Although increased glucose transport was associated with the activation of respiratory burst activity with PMA, fMLP and GM-CSF, IL-3 failed to stimulate superoxide production although it effectively promoted 2-DOG uptake. These results suggest that signalling pathways resulting from IL-3 ligation differ significantly from those of GM-CSF, despite shared βc receptor ‘signalling’ chains. Thus the α-chain of the GM-CSF receptor might impose respiratory burst specificity on βc signalling. Alternatively the GM-CSF α-chain might have signalling functions of its own. In this context it is interesting to note that the GM-CSF α-chain seems to be able to regulate glucose transport in *Xenopus* oocytes and in certain melanoma cell lines independently of βc [37,38].

Stimulation of 2-DOG uptake by PMA was independent of serum, whereas the effects of fMLP and Con A were dependent on serum. In contrast, IL-3- and GM-CSF-induced 2-DOG uptake by RAW cells was attenuated by serum. These results suggest that components in serum interact in a complex manner to modulate the ability of respiratory burst activators to promote glucose transport. The effects of serum on IL-3 responses of RAW cells are similar to those observed with 32Dcl23 [20].

Stimulation of 2-DOG uptake by PMA, fMLP and GM-CSF was correlated with respiratory burst activation but was unrelated to proliferative responses determined at 16 h, which were inhibited by PMA and fMLP but unaffected by GM-CSF. Therefore it seems that properties such as glucose transport that are essential for cell survival and proliferation are not tightly coupled to proliferative responses and might be under different regulatory controls, as indicated previously [39]. Longer periods of stimulation of cells with PMA, fMLP and Con A result in down-regulation of PKC, which is associated with the inhibition of proliferative responses. With IL-3-dependent 32D cells, cAMP promoted cell survival responses while inhibiting proliferative responses [40], an effect similar to that observed in RAW cells stimulated with PMA and fMLP. Because RAW cells are a macrophage tumour line not dependent on growth factors for survival and proliferation, it is not surprising that proliferative responses above those of control cells were not observed with GM-CSF and IL-3.

Enhanced glucose uptake by RAW cells was associated with an increased affinity of glucose transporters for glucose (lower K_m) without consistent changes in V_{max}. PMA, fMLP, IL-3 and GM-CSF all enhanced 2-DOG and 3-OMG uptake with a corresponding increase in transporter affinity for glucose. In other studies we have observed that the activation of human peripheral blood neutrophils by PMA results in increased affinity of Glut-1 for glucose (A. S. Tan and M. V. Berridge, unpublished work). Insulin-like growth factor 1 and IL-3 have also been shown to stimulate glucose transport in bone-marrow-derived cell lines [19,20]. In the present study, fMLP, IL-3 and GM-CSF had no significant effect on V_{max} for 2-DOG, whereas PMA decreased V_{max}. V_{max} is a complex function involving both cell surface expression and the intrinsic rate of transport. We have not established whether translocation is involved in the increased 2-DOG uptake observed in RAW cells but *de novo* synthesis can be excluded on the grounds that 15–60 min is insufficient time for transcription, translation and processing to occur.

To determine whether phosphorylation has a role in regulating transporter activation in response to PMA, fMLP and growth factors, inhibitors of tyrosine kinases (genistein), PKC (staurosporine and calphostin C) and PI 3-kinase (wortmannin) were used. Both genistein and staurosporine inhibited 2-DOG uptake by RAW cells and partly inhibited transporter activation. The cytoplasmic domain of Glut-3, the predominant transporter on RAW cells, exhibits a PKC consensus site but lacks tyrosine phosphorylation sites, indicating an indirect effect of genistein on glucose transport and a potential phosphorylation mechanism for acute regulation of Glut-3 activity. Although genistein has been shown to inhibit basal 2-DOG and 3-OMG uptake by interacting directly with Glut-1 on human erythrocytes, Chinese hamster ovary and leukaemic HL60 cells [41], in the present study preincubation with genistein for at least 40 min was necessary before inhibition was observed. Furthermore, before measuring 2-DOG and 3-OMG uptake, cells were washed to remove genistein, and uptake was determined in genistein-free medium.

Wortmannin had no effect on PMA-, fMLP- or growth-factor-induced glucose transport, suggesting that PI 3-kinases have no

role in the activation of glucose transport by these stimulants in RAW cells. These results are consistent with the lack of inhibition of cyanide-stimulated glucose transport by wortmannin in 3T3 adipocytes [42]. In contrast, wortmannin completely inhibited insulin-stimulated glucose transport in 3T3 cells [42] and abolished IL-3-stimulated glucose transport in 32D cells (N. Ahmed and M. V. Berridge, unpublished work), suggesting that multiple signalling cascades might be involved in the regulation of glucose transport in different cells. The lack of inhibition of glucose transport by wortmannin in RAW cells suggests that fMLP and GM-CSF might circumvent a requirement for PI 3-kinase in regulating glucose transport owing to constitutive activation of growth factor-induced pathways.

The possibility that cytosolic proteins might interact directly with glucose transporters in regulating transport activity has been considered. The activation of glucose transport in clone 9 fibroblasts by cyanide might involve association of cytosolic proteins with the cytoplasmic domain of Glut-1 [18], whereas other cytosolic proteins have been shown to be associated with the C-terminal domain of Glut-4 [43]. Moreover different hexokinase isoforms have been shown to be associated with different transporter subtypes [44]. The association of glyceraldehyde-3-phosphate dehydrogenase [45], aldolase [46] and glucokinase [47] with the human erythrocyte glucose transporter suggests that protein–protein interactions might have a crucial role in regulating transporter activity. These proteins could function by directly suppressing or activating the transporter, possibly by phosphorylation or appropriate association or dissociation, or could interfere with transporter association with an inhibitor molecule.

Our results show that agents that stimulate respiratory burst activity also enhance glucose uptake in RAW cells, suggesting that respiratory burst activation and glucose transport are linked and dependent on each other. Inhibitor studies suggest that the acute activation of glucose transport in RAW cells involves tyrosine and serine/threonine kinases but not PI 3-kinases. As well as phosphorylation-dependent activation, the possibility of the involvement of novel proteins that might intrinsically modulate transporter activity is under investigation.

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