Haemin enhancement of glucose transport in human lymphocytes: stimulation of protein tyrosine phosphatase and activation of p56^{lck} tyrosine kinase

Harry M. LANDER,*†§ Daniel M. LEVINE*† and Abraham NOVOGRODSKY*†‡||

*The Rogosin Institute and the Departments of †Biochemistry and ‡Medicine, Cornell University Medical College, 1300 York Avenue, New York, NY 10021, U.S.A.

Following our previous observation that haemin is mitogenic for human lymphocytes, we investigated the ability of haemin to enhance glucose uptake in these cells. We found that pre-incubation of human peripheral-blood mononuclear cells (PBMC) with haemin for 60 min increased up to 5-fold the rate of 2-deoxy-D-[1-³H]glucose uptake by the cells. Actinomycin D and cycloheximide did not inhibit the effect, and cytochalasin B completely blocked it. Among the metalloporphyrins tested (Fe-, Ni-, Co-, Zn- and Sn-protoporphyrin), only haemin (Fe-protoporphyrin) induced a marked increase in glucose uptake. Thiourea, a scavenger of oxygen free radicals, and 3-amino-1,2,4-triazole inhibited haemin-induced glucose uptake. Oxidants such as H₂O₂ and phenylarsine oxide were previously reported to stimulate protein tyrosine phosphorylation and to enhance glucose uptake. We found that incubation of PBMC with haemin

resulted in an increase in protein tyrosine phosphatase (PTPase) activity, probably that identified as CD45. Similarly to haemin, we found that phytohaemagglutinin also enhanced PTPase activity. Haemin also activated the tyrosine kinase p56^{lck}, which is negatively controlled by phosphorylation of Tyr-505 at the C-terminus, and increased protein tyrosine phosphorylation in these cells. Tyrphostins, specific inhibitors of tyrosine kinases, at low concentrations markedly enhanced glucose uptake and synergized with haemin in enhancing glucose uptake. At high doses, tyrphostins inhibited the effect of haemin. Taken together, we postulate that haemin enhancement of glucose uptake in human lymphocytes results from its stimulation of PTPase, followed by activation of tyrosine kinase p56^{lck}, leading to an increase in protein tyrosine phosphorylation.

INTRODUCTION

Mitogenically stimulated lymphocytes undergo a variety of metabolic responses. One of the earliest is an enhancement of glucose uptake [1]. The mitogens phytohaemagglutinin (PHA) [1], concanavalin A (Con A) [2] and phorbol myristic acetate [3] all have been shown to rapidly induce glucose uptake. More recently it has been shown that human peripheral-blood mononuclear cells (PBMC) have a translocatable intracellular pool of glucose transporters [4] which is recruited to the plasma membrane within 30–60 min of PHA stimulation. Glucose transport in lymphocytes is similar to that of adipocytes, in that it involves a facilitated-diffusion mechanism [1]. Oxidants have also been shown to induce glucose uptake in various cell types [5–7]. This is associated with a translocation of glucose transporters from a perinuclear region to the plasma membrane in BHK cells, similar to that seen in insulin-treated cells.

Oxidants were also reported to stimulate protein tyrosine phosphorylation [8], and inhibition of protein tyrosine phosphatase (PTPase) was implicated to mediate this effect [9]. Vanadate, a PTPase inhibitor, has been shown to mimic insulin activity by stimulating glucose uptake in adipocytes [9]. Activation of the insulin-receptor tyrosine kinase by insulin results in its autophosphorylation, followed by enhancement of glucose transport.

We have previously shown that haemin and other iron-containing compounds are mitogenic to human lymphocytes [10,11], and suggested that the oxidative properties of haemin

may mediate this effect. In the present study we investigated the effect of haemin on glucose uptake in human peripheral lymphocytes. We have tested the hypothesis that haemin-induced stimulation of PTPase, followed by activation of p56^{lck} tyrosine kinase, mediates the enhancing effect of haemin on glucose transport.

MATERIALS AND METHODS

Cell isolation and culture

Human PBMC were isolated from healthy volunteers by Ficoll–Hypaque density-gradient centrifugation. The murine lymphoma BW5147 (CD45⁺) and mutant BW5147(T200-a)5.1 (CD45[−]) cell lines were kindly given by Dr. Robert Hyman, The Salk Institute, San Diego, CA, U.S.A. The CD45[−] cells expressed less than 1 % of CD45 antigen in the membrane or cytoplasm [12,13]. These cells were maintained in Dulbecco's modified Eagle medium with high glucose (JRH Bioscience, Lenexa, KS, U.S.A.) containing 10 % heat-inactivated horse serum. All chemicals were purchased from Sigma (St. Louis, MO, U.S.A.), except the different protoporphyrins. The tyrphostins were provided by Dr. A. Levitzki (Hebrew University, Jerusalem, Israel), and stock solutions were dissolved to 50 mM in dimethyl sulphoxide. The final concentration of dimethyl sulphoxide in tissue culture was less than 0.1 %. Protoporphyrins (Porphyrin Products, Logan, UT, U.S.A.) were dissolved in 1 M NaOH, neutralized with 0.5 M Tris, pH 7.5, and diluted to 500 μM stock solutions.

Abbreviations used: PTPase, protein tyrosine phosphatase; PHA, phytohaemagglutinin; Con A, concanavalin A; PBMC, peripheral-blood mononuclear cells; TCR, T-cell antigen receptor.

§ To whom correspondence and reprint requests should be addressed

|| Present address: The Rogoff Institute, Beilinson Medical Center, Petah-Tikva, 49100, Israel.

2-Deoxy-D-[1-³H]glucose uptake

Fresh PBMC were resuspended to 10×10^6 cells/ml in PBS containing 1 mg/ml BSA and 5 mM lactate. After treatment, cells (0.5 ml per data point) were put on a shaker, and 10 μ l of 5 mM 2-deoxy-D-glucose containing 5 μ Ci of 2-deoxy-D-[1-³H]glucose (Amersham, Arlington Heights, IL, U.S.A.) was added. After 5 min, 200 μ l was removed, layered on top of dibutyl phthalate/mineral oil (4:1, v/v) and spun in a Microfuge for 30 s. The tip (with the cell pellet) was cut and placed in 1 ml of 1 % Triton X-100/0.1 M NaOH, left overnight and counted for radioactivity with 15 ml of Ultrafluor (National Diagnostics, Manville, NJ, U.S.A.) in a liquid-scintillation counter. The assay was linear at 5 min.

Preparation of particulate fraction

PBMC or murine lymphoma cells were washed in PBS and resuspended to approx. 40×10^6 cells/ml in 25 mM Hepes/5 mM sodium lactate containing 1 mg/ml BSA and divided into batches for treatment. After an appropriate incubation time, cells were washed twice with wash buffer (25 mM Hepes, pH 7.4, 5 mM KCl, 150 mM NaCl) and resuspended with 100 μ l of sonication buffer (25 mM Tris, pH 7.5, 1 mM MgCl₂, 1 mM phenylmethanesulphonyl fluoride, 100 μ g/ml aprotinin, 10 μ g/ml leupeptin). They were then sonicated in a bath sonicator (Laboratory Supplies, Hicksville, NY, U.S.A.) for 20 s and put on ice for 40 s. This was repeated ten times or until inspection of samples under a microscope showed complete disruption. The tubes were spun at 800 g for 5 min to pellet debris, and the supernatants were spun for 1 h at 100 000 g at 4 °C. The supernatants were discarded and the pellets resuspended in 100 μ l of sonication buffer by vortex-mixing. This particulate fraction was put on ice and assayed for protein by the Lowry method as modified by Markwell et al. [14].

PTPase assay

Particulate fractions were assayed as described by Streuli et al. [15] with ³²P-Raytide as substrate. Raytide (Oncogene Science, Manhasset, NY, U.S.A.), a gastrin analogue, was tyrosine-phosphorylated in the presence of [γ -³²P]ATP with the *v-abl* protein (Oncogene Science). ³²P-Tyr-Raytide was recovered by trichloroacetic acid precipitation with extensive washing to remove unincorporated label.

A 5 μ l portion of sample (0.1–0.5 mg/ml in sonication buffer) was mixed with 5 μ l of ³²P-Tyr-Raytide [(1–5) $\times 10^4$ c.p.m.] in 50 μ l of buffer (25 mM Hepes, pH 7.3, 50 mM EDTA, 100 mM dithiothreitol). Sample concentration was varied such that no more than 10 % of the substrate was destroyed by the enzyme during the 10 min incubation at 37 °C. To terminate, 750 μ l of acidic charcoal was added, tubes were centrifuged in a Microfuge for 5 min and 400 μ l of supernatant was counted for radioactivity in a liquid-scintillation counter. The assay was linear at 10 min. Data are expressed as percentages of control. One unit equals 1 pmol of P_i released/min per mg of protein.

H₂O₂ assay

A reaction mixture containing 50 mM sodium phosphate, pH 7.3, 20 μ M 3-amino-1,2,4-triazole and 17 units of bovine catalase (twice crystallized) in 30 μ l was mixed with 20 μ l of sample and incubated at 37 °C in a shaking water bath. After 1 h, 950 μ l of 82.1 mM Na₂BO₃, pH 6.8, was added and left shaking for another 5 min at 37 °C. Then 1 ml of 1 M H₂SO₄ was added to stop the reaction, and the undestroyed perborate was titrated with 50 mM

KMnO₄ (about 500 μ l). Data are expressed as percentage of perborate destroyed, and a linear regression of a standard curve of known H₂O₂ concentration was used to calculate unknowns.

Determination of protein tyrosine phosphorylation

PBMC (20×10^6 /ml in RPMI 1640 containing 5 % heat-inactivated fetal calf serum) were labelled with 1 mCi of [³²P]P_i/ml for 4 h. After treatment with haemin for 1 h, cells were lysed in 250 μ l of immunoprecipitation (IP) buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 3 % Nonidet P40, 10 mM MgCl₂, 1 mM phenylmethanesulphonyl fluoride, 0.4 mM vanadate, 1 mM molybdate, 200 Kunitz units of DNAase) containing 5 μ g of anti-phosphotyrosine antibody (PY20 clone; ICN Biomedicals, Costa Mesa, CA, U.S.A.). After 1 h, 25 μ l of a 1:1 suspension of Sepharose–Protein G was added and incubated for another 1 h. Immunoprecipitates were washed three times in IP buffer and electrophoresed on SDS/3–27 % polyacrylamide gels with a 3 % stacking gel. Electrophoresis was carried out at 100 V/gel until the dye front nearly ran off the gel. The gel was soaked in transfer buffer (0.05 M glycine, 0.05 M Tris, pH 8.8) for 15 min and electro-transferred on to 0.22 μ m-pore nitrocellulose paper for 22 h at 35 V at 4 °C. After transfer, the nitrocellulose was exposed to XOMAT film (Kodak, Rochester, NY, U.S.A.) for 16 h at –70 °C with intensifying screens. ¹⁴C-labelled protein standards (Amersham) were included on all gels to estimate molecular masses.

Assay of p56^{lck} activity and phosphoamino acid analysis

Activity of p56^{lck} was assessed both *in vitro* and *in vivo*. The assay *in vitro* was the immune-complex kinase assay as follows. PBMC (20×10^6 cells) were treated as indicated. Cells were pelleted, washed three times with PBS and lysed in 250 μ l of anti-CD4 buffer (91 % Nomdet P-40, 150 mM NaCl, 1 mM Na₂MoO₄, 0.4 mM Na₃VO₄, 5 mM MgCl₂, 1 mM phenylmethanesulphonyl fluoride, 10 μ g/ml leupeptin and 5000 units/ml DNAase). After 30 min at 4 °C, 5 μ g of anti-CD4 (OKT4; Ortho Diagnostics), which co-precipitates p56^{lck} [16], was added to each sample, and after another 1 h at 4 °C, 25 μ l of a 1:1 suspension of Sepharose–Protein G was added. After 1 h, samples were washed three times with anti-CD4 buffer, and 30 μ l containing 25 mM Hepes, 0.1 % Nonidet P40 and 10 μ Ci of [γ -³²P]ATP was added. After 10 min at 25 °C, 30 μ l of SDS sample buffer was added and samples were boiled. Samples were electrophoresed and transferred as described above.

The assay *in vivo* consisted of labelling whole cells with [³²P]P_i exactly as described for the determination of protein tyrosine phosphorylation above, except that 5 μ g of OKT4 (Ortho Diagnostics) antibody was added instead of anti-phosphotyrosine. After autoradiography, p56^{lck} was localized, excised and processed for one-dimensional phosphopeptide mapping by CNBr cleavage [17]. Excised bands were digested in 500 μ l of 100 mg/ml CNBr in 70 % formic acid for 1.5 h at 22 °C. Samples were freeze-dried, electrophoresed on an SDS/24 % acrylamide gel, and the gel was dried and exposed for 6 days to X-ray film. ¹⁴C-labelled low-molecular-mass standards (Amersham) were included on each gel.

RESULTS

Haemin-induced enhancement of glucose uptake in human and murine lymphocytes

Following our observation that haemin is mitogenic for human T lymphocytes, we have investigated the ability of haemin to enhance glucose uptake in human lymphocytes. Glucose uptake

Figure 1 Haemin-induced enhancement of 2-deoxy-D-[1-³H]glucose uptake

Human PBMC were incubated for 1 h with haemin, followed by measurement of the rate of 2-deoxy-D-[1-³H]glucose uptake as described in the Materials and methods section. The data represent means of duplicate determinations from two separate experiments. In these experiments, the rate of 2-deoxy-D-[1-³H]glucose uptake (mean $\pm$ S.D.) of control cells was 13.6 ± 1.2 pmol/min per 10^6 cells and was set equal to 100%.

is one of the early functions that were previously reported to be induced by a variety of mitogens [1–3]. As shown in Figure 1, incubation of PBMC with haemin for 60 min at 37 °C increased the rate of 2-deoxy-D-[1-³H]glucose uptake by the cells. The response curve was biphasic. Maximal stimulation was attained at 10 μ M haemin, and higher concentrations were less stimulatory. Haemin-induced enhancement of glucose uptake in human T lymphocytes depleted of macrophages was similar to that found in human PBMC (results not shown). In contrast, haemin-induced mitogenesis is macrophage-dependent [10]. This suggests that the late events of mitogenesis require additional signals that are not provided by haemin alone.

In addition, haemin (50 μ M) stimulated the rate of glucose uptake in a murine lymphoma cell line, BW5147, from 16.2 ± 2.1 to 27.9 ± 3.4 pmol/min per 10^6 cells. In contrast, a mutant of this cell line lacking CD45 was refractory to haemin-induced glucose uptake. Values for control and haemin-treated cells were 8.0 ± 1.2 and 8.6 ± 2.8 pmol/min per 10^6 cells respectively.

We have determined the time required for haemin to induce an increase in glucose uptake. Human PBMC were incubated with haemin for different time intervals, followed by glucose-uptake determinations. As shown in Figure 2, the haemin-induced increase in glucose uptake was time-dependent. Incubation of the cells with haemin for 60 min resulted in maximal glucose uptake. Incubation of PBMC with haemin in the presence of actinomycin D or cycloheximide did not abrogate the increase in glucose uptake (Table 1). These findings indicate that the effect of haemin in enhancing glucose uptake does not involve activation of transcriptional and translational mechanisms. In contrast with actinomycin D and cycloheximide, cytochalasin B, an inhibitor of the glucose transporter, completely blocked the haemin-induced increase in glucose uptake (Table 1).

Induction of glucose uptake by different metalloporphyrins

We have evaluated the effect of different metalloporphyrins and protoporphyrin IX on their ability to induce glucose uptake in lymphocytes. As shown in Table 2, among the metalloporphyrins tested (i.e. Fe-, Ni-, Co-, Zn- and Sn-protoporphyrins), only haemin (Fe-protoporphyrin) induced a marked increase in glucose uptake, Co- and Zn-protoporphyrin were slightly active, and Sn-protoporphyrin was inhibitory for basal glucose uptake.

Figure 2 Time course of haemin-induced 2-deoxy-D-[1-³H]glucose uptake in human lymphocytes

Human PBMC were incubated with or without 30 μ M haemin for the indicated times, followed by measurement of the rate of 2-deoxy-D-[1-³H]glucose uptake as described in the Materials and methods section. The results represent means of duplicates from a single experiment. Additional experiments gave similar values.

Table 1 Effect of metabolic inhibitors on haemin-induced 2-deoxy-D-[1-³H]glucose uptake

Human PBMC were incubated for 1 h in the absence or presence of haemin and various inhibitors, followed by measurement of the rate of 2-deoxy-D-[1-³H]glucose uptake as described in the Materials and methods section

Treatment	Glucose uptake (pmol/min per 10^6 cells)
None	20 ± 3.4
Haemin (30 μ M)	104 ± 8.7
Haemin (30 μ M) + cytochalasin B (10 μ M)	24 ± 2.9
Haemin (30 μ M) + cycloheximide (20 μ M)	96 ± 8.1
Haemin (30 μ M) + actinomycin D (10 μ M)	115 ± 9.8

Ni-protoporphyrin and protoporphyrin IX had no activity. We have considered the possibility that the oxidative properties of haemin are responsible for its stimulating effect on glucose uptake, and measured the generation of H_2O_2 catalysed by haemin and the different metalloporphyrins. As shown in Table 2, haemin, Co- and Zn-protoporphyrins are potent generators of H_2O_2 , whereas Ni- and Sn-protoporphyrins are poor generators. We have previously shown that the oxygen free-radical scavenger thiourea and the peroxidase inhibitor 3-amino-1,2,4-triazole inhibit haemin-induced lymphocyte activation [10,18]. We have found that both agents at concentrations that inhibit haemin-induced mitogenesis also inhibited haemin enhancement of glucose uptake (results not shown). 2-Mercaptoethanol and dithiothreitol at concentrations up to 1 mM did not inhibit the haemin-induced increase in glucose uptake (results not shown).

Effect of haemin on PTPase activity in human PBMC

Oxidants such as phenylarsine oxide and H_2O_2 were previously reported to stimulate glucose uptake in a variety of cell types [5–7]. Inhibition of PTPase activity by these agents, which led to an increase in PTyr-P, was implicated to play a role in this process. We therefore explored the possibility that haemin, a known oxidant, increased glucose uptake in human PBMC by a similar mechanism.

Table 2 Effect of haemin analogues on 2-deoxy-D-[1-³H]glucose uptake and H₂O₂ production in human lymphocytes

Human PBMC were incubated for 1 h with 30 μ M of the indicated haemin analogue, followed by assay of the rate of 2-deoxy-D-[1-³H]glucose uptake. H₂O₂ production was measured in a cell-free system as described in the Materials and methods section. 'IX' indicates protoporphyrin IX. The data represent means of duplicate determinations from two separate experiments.

Protoporphyrin	Glucose uptake (pmol/min per 10 ⁶ cells)	H ₂ O ₂ generated (μ M)
None	18.1 $\pm$ 2.3	None detected
Fe	95.3 $\pm$ 9.1	425 $\pm$ 12
Ni	18.6 $\pm$ 2.8	37 $\pm$ 6
Co	20.8 $\pm$ 2.4	423 $\pm$ 17
Zn	21.2 $\pm$ 3.6	261 $\pm$ 14
Sn	12.8 $\pm$ 2.7	92 $\pm$ 8
IX	17.6 $\pm$ 3.0	None detected

Figure 3 Effect of haemin on PTPase activity in human lymphocytes and murine mutant cells

Human PBMC, parental murine lymphoma CD45⁺ or mutant CD45⁻ cells were incubated for 1 h with haemin, followed by measurement of PTPase activity in the particulate fraction as described in the Materials and methods section. The data represent means of triplicate determinations from two separate experiments. In these experiments, PTPase activity (mean $\pm$ S.D.) of control CD45⁺ cell membranes was 0.045 $\pm$ 0.002 unit and that of control CD45⁻ cell membranes was 0.022 $\pm$ 0.003 unit, and both were set equal to 100%.

Haemin was incubated with human PBMC, followed by disruption of the cells by sonication, and PTPase activity in the particulate fraction was assayed.

Surprisingly, we have found that haemin at concentrations of 30–100 μ M stimulated PTPase activity in human PBMC (Figure 3). CD45 (leucocyte common antigen) is expressed in cells of the haematopoietic lineage and exhibits PTPase activity. We have found that haemin also stimulated PTPase activity in a murine lymphoma cell line, but failed to do so in a mutant lacking CD45 (Figure 3).

Effect of PHA on PTPase activity in human PBMC

Mitogens such as PHA were previously reported to enhance glucose transport and protein tyrosine phosphorylation in various cell types. We have investigated whether PHA shares with haemin its ability to activate PTPase activity in human PBMC. We have found that treatment of PBMC for 1 h with PHA (1 μ g/ml) led to an increase in PTPase activity from

Figure 4 Tyrphostins induce 2-deoxy-D-[1-³H]glucose uptake in human lymphocytes

Human PBMC were incubated for 1 h with each tyrphostin, followed by measurement of the rate of 2-deoxy-D-[1-³H]glucose uptake as described in the Materials and methods section. The structure of each tyrphostin is shown. The data represent means of duplicate determinations from a single experiment. Similar results were seen in several other experiments. In this experiment, the rate of 2-deoxy-D-[1-³H]glucose uptake (mean $\pm$ S.D.) of control cells was 14.7 $\pm$ 3.3 pmol/min per 10⁶ cells and was set equal to 100%.

0.068 $\pm$ 0.007 unit in membranes from untreated cells to 0.092 $\pm$ 0.011 unit in membranes from PHA-treated cells.

Effect of tyrphostins on glucose uptake in human PBMC

In view of the possibility that increased PTPase activity mediated the enhancing effect of haemin on glucose uptake in lymphocytes, we investigated whether inhibitors of protein tyrosine phosphorylation may mimic the effect of haemin on glucose uptake. We found that tyrphostins, specific inhibitors of tyrosine kinases [19–22], markedly enhance glucose uptake in lymphocytes (Figure 4). The response curve was biphasic and characteristic for each of the tyrphostins tested. The most potent inducer of glucose uptake was AG17, which maximally stimulated at a concentration of 1 μ M.

Tyrphostin AG126 at low concentrations was found to synergize with haemin in enhancing glucose uptake. Higher concentrations of the tyrphostin inhibited the stimulatory activity of haemin (Figure 5).

Haemin activation of the tyrosine kinase p56^{lck}

Oxidants such as H₂O₂ and phenylarsine oxide were shown to enhance glucose uptake and to stimulate protein tyrosine phosphorylation in several cell types [8]. We examined the possibility that stimulation of tyrosine phosphorylation may mediate the enhancing effect of haemin on glucose uptake in human PBMC.

The *src*-family protein-tyrosine kinases are plasma-membrane-associated proteins which have been shown to participate in lymphocyte signalling [23]. p56^{lck} is of particular interest because

Figure 5 Haemin and tyrphostins synergistically induce 2-deoxy-D-[1-³H]glucose uptake

Human PBMC were treated with haemin in the absence or presence of the indicated concentration of tyrphostin AG126. The rate of 2-deoxy-D-[1-³H]glucose uptake was measured as described in the Materials and methods section. The data represent means of duplicate determinations from a single experiment. Similar results were seen in another experiment. In this experiment, the rate of 2-deoxy-D-[1-³H]glucose uptake (mean $\pm$ S.D.) of control cells was 14.1 ± 2.2 pmol/min per 10^6 cells and was set equal to 100%.

Figure 6 Effect of haemin on p56^{lck} kinase activity

Human PBMC were treated with the indicated concentrations of haemin for 1 h before analysis of immune-complex kinase activity as described in the Materials and methods section.

Figure 7 Effect of haemin on p56^{lck} autophosphorylation *in vivo*

Human PBMC were labelled with [³²P]P_i and treated with the indicated concentration of haemin for 1 h, followed by analysis of p56^{lck} phosphorylation as described in the Materials and methods section. (a) SDS/PAGE of OKT4 immunoprecipitates, and (b) of the corresponding peptides produced by CNBr cleavage.

Figure 8 Effect of haemin on protein tyrosine phosphorylation

Human PBMC were labelled with [³²P]P_i and then treated with the indicated concentrations of haemin for 1 h or pre-treated with tyrphostin AG126 for 15 min before immunoprecipitation with anti-phosphotyrosine antibody and analysis of protein tyrosine phosphorylation as described in the Materials and methods section.

it is predominantly expressed in T lymphocytes. This enzyme autophosphorylates Tyr-394 upon activation [24,25] and is negatively regulated by tyrosine phosphorylation of its C-terminus at Tyr-505. Thus p56^{lck} could be activated by an agent such as haemin which stimulates PTPase activity. Two assays were employed to demonstrate the activation of p56^{lck} by haemin.

First, cells were treated with haemin and the ability of immunoprecipitated p56^{lck} to autophosphorylate itself *in vitro* was analysed (Figure 6). In the second assay, PBMC were labelled with [³²P]P_i, then treated with haemin and immunoprecipitated p56^{lck} was analysed for autophosphorylation at Tyr-394 (Figure 7). The appearance of a 9 kDa fragment of p56^{lck} upon CNBr cleavage is indicative of Tyr-394 autophosphorylation [24]. Hence, both assays indicated that p56^{lck} was activated by haemin.

Treatment of PBMC with haemin also increased tyrosine phosphorylation of multiple proteins (Figure 8). This was demonstrated by labelling the cells with [³²P]P_i, followed by immunoprecipitation with anti-phosphotyrosine antibody, electrophoresis and autoradiography. As shown in Figure 8, tyrphostin AG126 at low dose (2 μM) potentiated the effect of haemin and at high dose (50 μM) inhibited it. Interestingly, the effect of AG126 on haemin-induced protein tyrosine phosphorylation is similar to its effect on haemin-induced glucose uptake, namely agonistic at low doses and antagonistic at high doses.

In contrast with haemin, tyrphostin AG126 at concentrations of 2 μM and 10 μM that enhance glucose uptake did not activate the tyrosine kinase p56^{lck}, as determined by the two assays outlined above. Tyrphostin AG126, at a concentration of 50 μM that inhibits haemin enhancement of glucose uptake, completely inhibited haemin activation of the tyrosine kinase p56^{lck}.

DISCUSSION

One of the earliest metabolic events that are induced in lymphocytes upon mitogenic stimulation is an increase in glucose transport [1]. Oxidants such as H₂O₂ and phenylarsine oxide were previously shown to enhance glucose uptake in different cell types [5–7]. Following our observation that haemin, a known oxidant, is mitogenic for human peripheral lymphocytes, we studied the effect of haemin on glucose uptake by the cells.

Incubation of human PBMC with haemin for 60 min increased up to 5-fold the rate of glucose uptake. The response curve had a bell shape, i.e. high concentrations of haemin were less stimulatory. The enhancing effect of haemin on glucose uptake involved the glucose transporter, since cytochalasin B, known to inhibit the glucose transporter, completely abrogated the haemin effect.

We have considered the possibility that the oxidative properties of haemin mediate its enhancing effect on glucose uptake. Among the metalloporphyrins tested, only haemin (Fe-protoporphyrin) had a marked stimulatory effect, whereas Co- and Zn-protoporphyrins were slightly stimulatory. On the other hand, Fe-, Co- and Zn-protoporphyrins were essentially similar in their ability to generate H_2O_2 . Thus, quantitatively, the correlation between the ability of metalloporphyrins to generate H_2O_2 *in vitro* and their ability to enhance glucose uptake is not apparent. It is possible that, in addition to its ability to generate H_2O_2 , haemin has to bind to an appropriate site in the cell in order to elicit its stimulatory effect of glucose uptake (i.e. an oxidation-independent mechanism). This property may not be shared by Co- and Zn-protoporphyrins. The observation that thiourea, an oxygen free-radical scavenger, and 3-amino-1,2,4-triazole, an inhibitor of peroxidase activity, blocked haemin-induced glucose uptake supports the notion that oxidative mechanisms are involved in the haemin-induced increase in glucose uptake.

Oxidants such as H_2O_2 and phenylarsine oxide were previously shown to exhibit insulinomimetic properties, namely stimulation of protein tyrosine phosphorylation and enhancement of glucose transport [8]. Oxidant inhibition of PTPase was implicated in mediating their ability to enhance tyrosine phosphorylation [9].

Treatment of human PBMC with haemin, over a broad range of concentrations, resulted in enhancement of PTPase activity. Similar effects were observed using a murine lymphoma cell line (wild-type CD45⁺). In contrast, haemin did not stimulate, but rather inhibited, PTPase activity in a mutant lacking CD45 (CD45⁻). These findings suggest that CD45, the major PTPase in T lymphocytes, is stimulated by haemin. In fact, we found that the rate of glucose uptake was enhanced in wild-type cells treated with haemin, but not in the mutant cells.

The question of whether the signals induced by stress stimuli are unique or similar to mitogens known to interact with the T-cell antigen receptor (TCR)-CD3 complex was investigated. We found that PHA and Con A treatment of human lymphocytes led to enhanced PTPase activity. In addition, these lectins are known to share with stress stimuli other properties, that is the ability to enhance rapidly glucose uptake and protein tyrosine phosphorylation. Antigens as well as mitogenic lectins are known to ligate and induce redistribution of membrane sites, the most notable of which is the TCR-CD3 complex, leading to a cascade of structural and biochemical events. One such event is the interaction of CD45 with $p56^{lck}$, which may lead to activation of $p56^{lck}$ kinase activity. We have observed an increase in PTPase activity upon mitogenic stimulation. It is possible that interaction between CD45 and its substrate, $p56^{lck}$, leads to enhanced PTPase activity. Alternatively, PTPase activity could be enhanced before the association by a mechanism as yet unidentified.

In contrast with antigens, the primary structure or structures affected by haemin are unknown. Haemin may induce redistribution of membrane sites, similar to that induced by antigens and mitogens, possibly by affecting cytoskeletal components. In fact, we found that haemin induced capping in murine splenocytes and induced actin polymerization in human PBMC [25a]. It is possible that haemin activates CD45 by a mechanism distinct from that of mitogens. CD45 may be indirectly activated by oxidation of a susceptible thiol moiety on a negative regulator of

CD45, whose inactivation may lead to enhanced CD45 activity. It should be noted, however, that lymphocyte PTPase (CD45) has a thiol moiety that is essential for its catalytic activity [26], and direct oxidation of the enzyme may result in its inhibition.

CD45 was implicated to play a role in activation of the T-lymphocyte tyrosine kinase $p56^{lck}$ [27], which is negatively controlled by phosphorylation of Tyr-505 at its C-terminus. We have demonstrated in this study, using two different assays, that $p56^{lck}$ was activated in haemin-treated PBMC. In addition, we have also shown that haemin stimulated protein tyrosine phosphorylation, and it is reasonable to assume that these two haemin-induced effects are related.

We have found that tyrphostins, specific inhibitors of tyrosine kinases [19–22], at low concentration mimic the effect of haemin in enhancing glucose transport. Activation of $p56^{lck}$ can occur in two ways: either through dephosphorylation of Tyr-505 by CD45 (or another tyrosine phosphatase), or by prevention of its phosphorylation by an unknown tyrosine kinase. We have recently proposed [28] that several tyrphostins exert their potent agonistic properties on resting human PBMC through this latter mechanism. Tyrphostin AG126 at concentrations that enhance glucose uptake failed to activate tyrosine kinase $p56^{lck}$. It is possible that tyrphostins mediate their enhancing effect on glucose transport by activation of other tyrosine kinases which are negatively controlled by tyrosine phosphorylation, such as *c-src* or *fyn*. Another possibility is that the tyrphostin effect is mediated by inhibiting tyrosine phosphorylation of a regulatory protein that affects the glucose transporter.

The cascade of events leading from modulation of protein tyrosine phosphorylation to translocation of the glucose transporter are not yet resolved. Recent data [4] suggest that treatment of human PBMC with PHA leads to a rapid translocation of glucose transporters with medium-affinity binding sites. Because PHA and haemin share the ability to enhance PTPase activity and glucose uptake, it will be of interest to identify the molecular targets in the protein tyrosine-phosphorylation signal-transduction pathway involved in translocation of the glucose transporter.

In addition, recent studies indicate that turnover of the phosphate groups of pp15, a product of insulin-receptor tyrosine kinase action, is coupled to signal transmission of the glucose-transport system [29]. It is important to note that this system involves hepatocyte insulin-induced glucose uptake, a distinct type of glucose transport from that discussed here. The possible involvement of haemin-enhanced PTPase in this mechanism leading to enhancement of glucose transport should also be considered.

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