

Activation of G-protein coupled fMLP or PAF receptor directly triggers glucose transporter type 1 (GLUT1) translocation in Chinese hamster ovary (CHO) cells stably expressing fMLP or PAF receptor

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Abstract: The chemoattractants, fMLP and PAF, stimulate glucose uptake in phagocytes to obtain an energy source for host defense. Glucose uptake in phagocytes is mainly regulated via glucose transporter type 1 (GLUT1). To examine molecular mechanisms of facilitated glucose uptake in response to fMLP or PAF, we established CHO cells stably expressing fMLP or PAF receptor with c-myc epitope tagged GLUT1 which could immunologically detect GLUT1 on the cell surface. In the CHO cells, both fMLP and PAF directly triggered GLUT1 translocation from the intracellular pool to the cell surface, and stimulated glucose uptake. Therefore, in phagocytes, we propose that fMLP and PAF also trigger GLUT1 translocation to stimulate glucose uptake as an energy source for host defense. *J. Med. Invest.* 47 : 19-28, 2000

Key words: fMLP ; PAF ; glucose transport ; GLUT1 ; G-protein coupled receptor

INTRODUCTION

Phagocytes migrate across the endothelium along a chemoattractant gradient to the site of infection where they phagocytize invading microorganisms. Typical chemoattractants are bacterial derived formyl-peptides (e.g. formyl-methionyl-leucyl-phenylalanine (fMLP)), activated complements (C5a, C3a) and CXC chemokines (e.g. IL-8). Receptors against the chemoattractants are heptahelical ones coupled to pertussis toxin (*Bordetella pertussis* islet-activating protein (IAP)) sensitive heterotrimeric G-protein (Gi) (1, 2). Platelet-activating factor (PAF) also activates phagocytes via its receptor which is coupled to Gq- and Gi-protein. Binding of the chemoattractant to its receptor in phagocytes induces rapid activation of chemotaxis, respiratory burst, phagocytosis, and degranulation, the result being host defense.

There are two known major types of phagocytes ; polymorphonuclear leukocytes (PMNs) and cells of the monocyte-macrophage series. Phagocytes require extracellular glucose as an energy source for defense-related activities (3, 4).

Glucose uptake across the plasma membrane of mammalian cells is mediated by a family of integral membrane glycoproteins, namely glucose transporters. Glucose uptake is mainly regulated by the number of glucose transporters present on the cell surface (5). There are at least seven types of glucose transporters with different intracellular distributions despite similar structures (5). In insulin-responsive tissues such as adipose tissues and muscle, glucose transporter type 4 (GLUT4) is mainly expressed in the intracellular region under basal conditions. In response to various agents such as insulin, GLUT4 is rapidly translocated to the plasma membrane

Abbreviations : fMLP, formyl-methionyl-leucyl-phenylalanine ; IAP, *Bordetella pertussis* islet-activating protein ; PAF, platelet-activating factor ; PMN, polymorphonuclear leukocyte ; GLUT1, glucose transporter type 1 ; CHO cell, Chinese hamster ovary cell ; GLUT1myc, c-myc epitope-tagged GLUT1 ; FBS, fetal bovine serum ; 2-DG, 2-deoxy-D-glucose ; TGB, thioglycolate broth ; PMA, phorbol 12-myristate 13-acetate ; PI 3-kinase, phosphatidylinositol 3-kinase ; PDK, 3-phosphoinositide-dependent protein kinase ; MAPK, mitogen-activated protein ; ERK, extracellular signal-regulated

kinase ; PLC, phospholipase C ; IP₃, inositol 1,4,5-triphosphate ; DAG, diacylglycerol ; PKC, protein kinase C.

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resulting in stimulation of glucose uptake (5). In contrast, GLUT1 is ubiquitously expressed in many tissues and resides mainly on the cell surface to contribute to basal glucose uptake (5). A significant amount of GLUT1 remains in the intracellular pool and is translocated to the cell surface on the stimulation of insulin, but the degree of translocation is less than that for GLUT4 (6, 7). It is technically difficult to estimate the amount of endogenous GLUT1 translocated to the cell surface of phagocytes.

We developed a highly sensitive and quantitative method to detect directly and immunologically the translocation of glucose transporters on the cell surface of intact cells, using a c-myc epitope-tagged glucose transporter (8). Using this method, the translocation of GLUT1 and GLUT4 to the cell surface was induced by stimulation with insulin or other reagents in Chinese hamster ovary (CHO), 3T3-L1 and NIH 3T3 cells (7, 9). Recently, we found that activation of receptor-coupled heterotrimeric G-protein triggered GLUT4 translocation from intracellular pools to the cell surface in CHO cells, 3T3-L1 adipocytes, and L6 myotubes (10, 11). In the present study, we examined whether activation of G-protein coupled fMLP or PAF receptors would directly trigger GLUT1 translocation using c-myc epitope-tagged GLUT1 (GLUT1myc).

MATERIALS AND METHODS

Materials

Cell culture media and fetal bovine serum (FBS) were purchased from Gibco BRL (Rockville, MD). 2-[³H]-deoxy-D-glucose (2-DG) was from Moravsek Biochemicals Inc. (Brea, CA). Other reagents were of analytical grade from Sigma Chemical Co. (St. Louis, MO) and Wako Pure Chemicals Co. (Osaka, Japan).

Preparation of PMNs and macrophages

Specific pathogen-free Sprague-Dawley rats (5–7 wk old, male) were purchased from Charles River Japan Inc. (Yokohama, Japan), maintained under a 12-hour light-dark cycle at a constant temperature of 22 ± 2 °C and acclimatized for at least 1 week before the start of experiments. The rats were injected i.p. with 15 ml of thioglycolate broth (TGB) and killed 8–12 hr later following anesthetization with ether (12). The peritoneal cavities were incised and cells obtained by repeated lavage with PBS(-).

The cells were centrifuged. This was followed by NH₄Cl lysis of residual red blood cells, and two washes with RPMI 1640. Finally, the cells (PMNs) were suspended in RPMI 1640 containing 1% FBS at 4 °C, until use.

Rat peritoneal macrophages were isolated from rats injected with TGB 4 days before, using the same procedures (13). Cells from washings of rat peritoneal cavities were seeded on a 24-well plate in RPMI 1640 containing 10% FBS, and cultured for 48 hr. Adherent cells (macrophages) were assayed.

Establishment of stable cell lines specifically expressing G-protein coupled receptors

CHO cells were grown in Ham's F12 medium supplemented with 10% FBS. The parent cell line used in this study was CHO-GLUT1myc, a CHO cell line expressing GLUT1myc constructed by inserting a human c-myc epitope (14 amino acids) into the first ectodomain of GLUT 1 (8). The human fMLP receptor and PAF receptor were subcloned into a mammalian expression vector, pCXN2 (14–16). These plasmids were cotransfected into CHO-GLUT1myc cells with pSV2-*bs*, a blastcidin S deaminase expression plasmid, and selected with blastcidin S hydrochloride. Several independent clones expressing each receptor were established and designated as follows; CHO-GLUT1myc-fMLP•R and CHO-GLUT1myc-PAF•R were CHO-GLUT1myc cells stably expressing the fMLP receptor and PAF receptor, respectively.

Assay for 2-DG uptake by phagocytes and CHO cells

The PMNs (5 × 10⁶ cells) were suspended in 400 μl of Hank's balanced salt solution (glucose free) and pre-incubated at 37 °C for 30 min. PMNs were stimulated by addition of 50 μl of stimuli at a given concentration for an appropriate period. 2-DG uptake was measured as described (17).

The macrophages or the CHO-GLUT1myc cells in 24-well plates were incubated in 1 ml of KRH buffer (136 mM NaCl/4.7 mM KCl/1.25 mM CaCl₂/1.25 mM MgSO₄/20 mM Hepes (pH 7.4)/0.2% BSA) for 30 min at 37 °C, then in 300 μl of various concentrations of ligand-KRH solutions for appropriate periods at 37 °C. 2-DG uptake was measured as described (18).

Cell surface anti-c-myc antibody binding assay (GLUT1 translocation assay)

The CHO-GLUT1myc cells expressing various

receptors in 24-well plates were stimulated with their ligands, similar to the 2-DG uptake assay. GLUT1myc translocation was measured after fixation with 2% paraformaldehyde, as described (11).

Detection of Akt kinase activation

Cells were preincubated for 30 min in KRH buffer, and lysed in Laemmli's sample buffer (19) after treatment with the reagents for given periods at 37 °C. Cell lysates (10 µg) were analyzed using an anti-Akt antibody and ECL (enhanced chemiluminescence) system, after separation in 7% SDS-PAGE gels. The anti-Akt antibody was prepared by immunizing a rabbit with a C-terminal peptide (SLELQRTHFPQF SYASIRE) of rat Akt-2 (20). The phosphorylated Akt (Akt-P) showed slower migration than the unphosphorylated Akt (Akt) on the gel.

Statistical analysis

All data are expressed as the mean ± S.E. for a minimum of two separate experiments done in triplicate. Statistical analysis was conducted using Dunnett's test.

RESULTS

fMLP and PAF stimulate glucose uptake in phagocytes

fMLP, a chemotactic tripeptide, and phorbol 12-myristate 13-acetate (PMA) both stimulate glucose uptake in human peripheral blood PMNs and monocytes (21-23). Rat peritoneal PMNs and macrophages elicited by TGB-stimulation were isolated, and we confirmed that treatment of fMLP or PAF increased glucose uptake in these phagocytes, using 2-[³H]-deoxy-D-glucose as a tracer. Both fMLP and PAF significantly increased glucose uptake in PMNs, with maximal responses observed at 10 min (Fig. 1). At this time, 1 × 10⁻⁷ M fMLP and PAF stimulated glucose uptake by 31% (p<0.01) and 26% (p<0.01), respectively. In TGB-elicited rat peritoneal macrophages, PAF but not fMLP significantly stimulated glucose uptake, in a dose-dependent manner (Fig.2). Treatment with 1 × 10⁻⁷ M PAF for 15 min (maximum responses) stimulated glucose uptake by 84% (p<0.01). The lack of responsiveness for fMLP in rat macrophages is probably due to a lack of receptors (24). In PMNs, the treatment of fMLP or PAF also induced an immediate increase in glycogen phosphorylase a activity (data not shown). PMNs activated by fMLP or PAF make

Polymorphonuclear Leukocytes (PMNs)

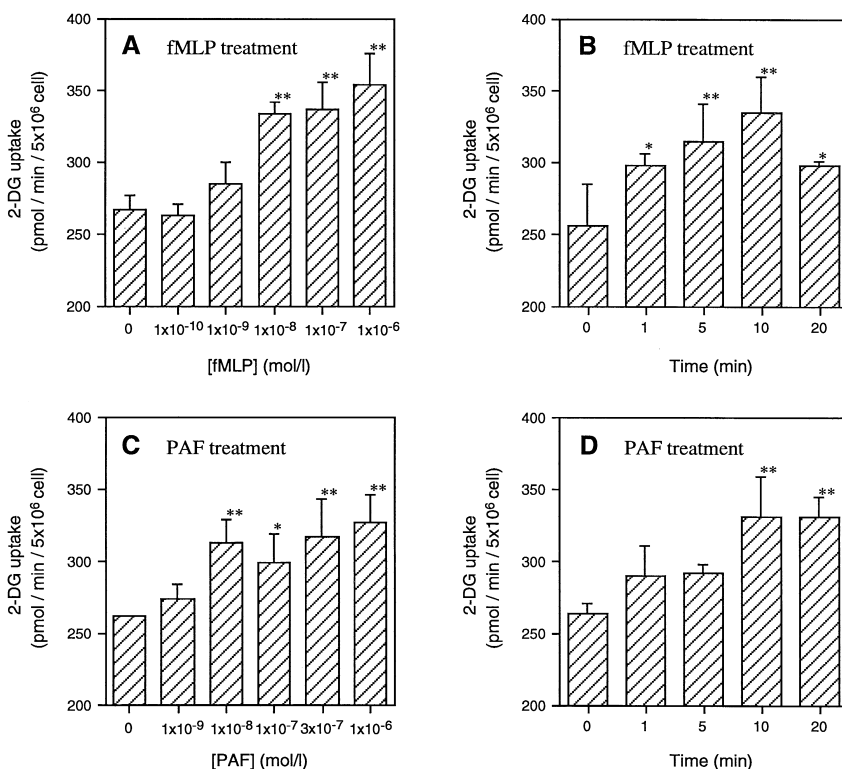


Fig.1. Effects of fMLP and PAF on 2-DG uptake by rat peritoneal PMNs. Rat peritoneal PMNs were stimulated with various concentrations of fMLP (A) or PAF (C) for 10min at 37 °C, or with 1 × 10⁻⁷ mol/l fMLP (B) or PAF (D) for indicated periods at 37 °C, and 2-DG uptake was determined. Values are the mean ± S.E. (n=6). Significantly different from the respective basal values : *p<0.05, **p<0.01 (Dunnett's test).

Macrophages

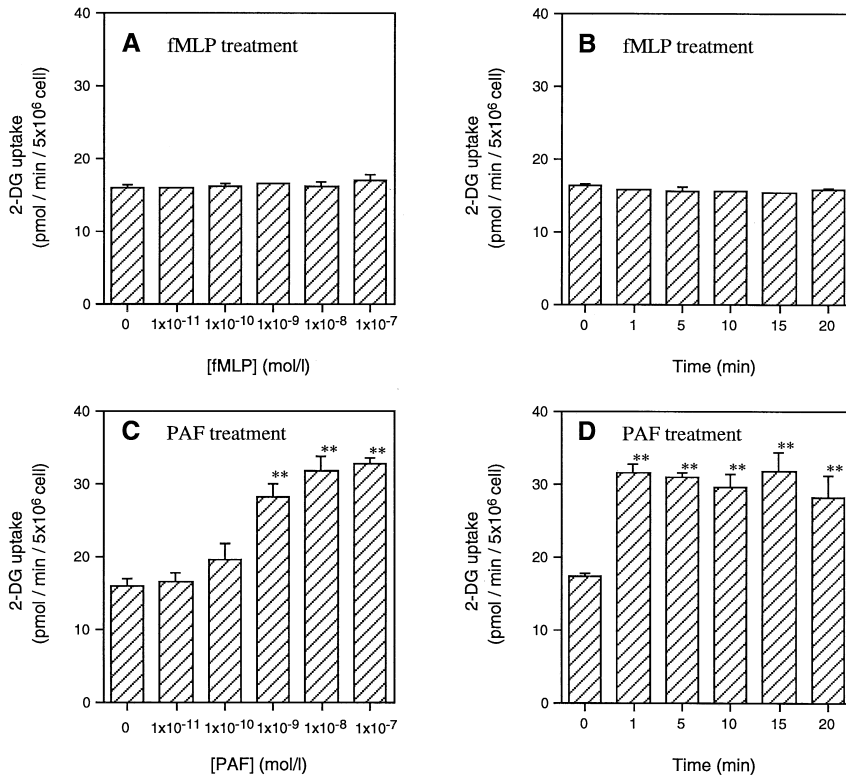


Fig. 2. Effects of fMLP and PAF on 2-DG uptake by rat peritoneal macrophages. Rat peritoneal macrophages were stimulated with various concentrations of fMLP (A) or PAF (C) for 10 min at 37 °C, or with 1 × 10⁻⁷ mol/l fMLP (B) or PAF (D) for indicated periods at 37 °C, and 2-DG uptake was determined. Values are the mean ± S.E. (n=6). **Significantly different from the respective basal values: p<0.01 (Dunnett's test).

use of endogenous glycogen as well as exogenous glucose as an energy source (4).

Human PMNs express the glucose transporter subtype, GLUT1, with no detectable GLUT3 (21, 25). TGB-elicited murine macrophages expressed GLUT1 mRNA but lacked the GLUT 2-5 isoforms determined using Northern analysis, as reported by Fukuzumi *et al* (14). Malide *et al* reported a change in the glucose transporter isoforms during the course of differentiation from monocyte to macrophage in cultured human monocyte-derived macrophages (26); they found that GLUT1 was present throughout the culture period, that GLUT3 and GLUT5 were present only at early and late differentiation stages, respectively, and that GLUT2 and 4 were not present at all. Therefore, regulation of GLUT1 translocation is most important for the early process of glucose uptake in phagocytes in response to fMLP or PAF. We also confirmed the expression of GLUT1 mRNA in rat TGB-elicited PMNs and macrophages, using RT-PCR (data was not shown).

fMLP or PAF stimulated GLUT1 translocation in CHO cells

We reported that insulin triggers GLUT1 trans-

location in 3T3-L 1 adipocytes and CHO cells stably expressing GLUT1myc, but that the degree of the translocation is less than that for GLUT4 (8). Since it is technically difficult to estimate the amount of endogenous GLUT1 translocated to the cell surface in phagocytes, we used CHO cells stably expressing GLUT1myc and chemoattractant-receptors as a model system to study the regulations of GLUT1 translocation and glucose uptake in response to chemoattractants. As shown in Fig. 3 A and C, in CHO cells stably expressing both GLUT1myc and fMLP receptors (CHO-GLUT1myc-fMLP•R), fMLP triggered GLUT1myc translocation in a dose- and time-dependent manner, whereas in the parent CHO cells expressing GLUT1myc (CHO-GLUT1myc), there was no response to fMLP. fMLP treatment also increased the rate of glucose uptake in CHO-GLUT1myc-fMLP•R cells, in proportion to GLUT1myc translocation (Fig. 3B, D). In CHO-GLUT1myc cells stably expressing PAF receptors (CHO-GLUT1myc-PAF•R), PAF treatment also triggered GLUT1myc translocation and stimulated glucose uptake (Fig. 4). Almost the same dose- and time-dependent GLUT1myc translocation and glucose uptake in response to fMLP or PAF were observed. These findings suggest that fMLP- and PAF-stimulated glucose uptake is

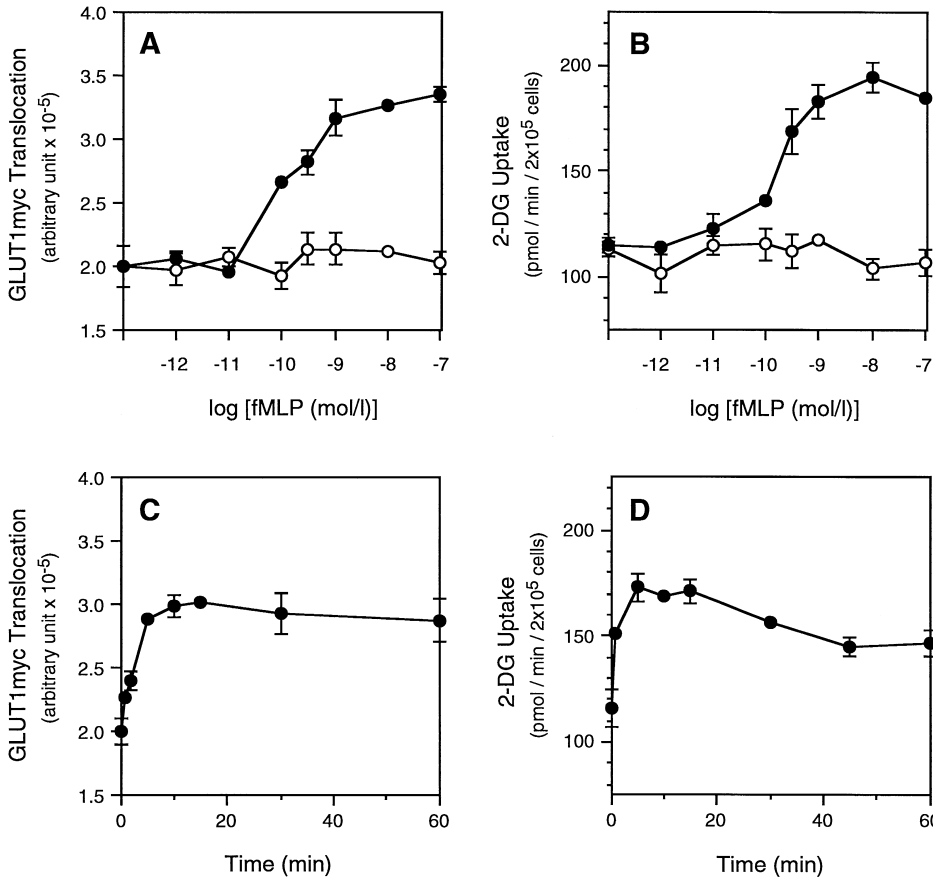


Fig. 3. Dose- and time-dependent GLUT1myc translocation and 2-DG uptake in response to fMLP in CHO-GLUT1myc-fMLP•R cells. A and B, the parent cells (CHO-GLUT1myc cells) (○) and cells expressing fMLP•R (CHO-GLUT1myc-fMLP•R cells) (●) were stimulated with various concentrations of fMLP for 10 min at 37 °C. The GLUT1myc translocation (A) and 2-DG uptake (B) are shown. C and D, CHO-GLUT1myc-fMLP•R cells were stimulated with 1 × 10⁻⁷ mol/l fMLP for the indicated periods at 37 °C. The GLUT1myc translocation (C) and 2-DG uptake (D) are shown.

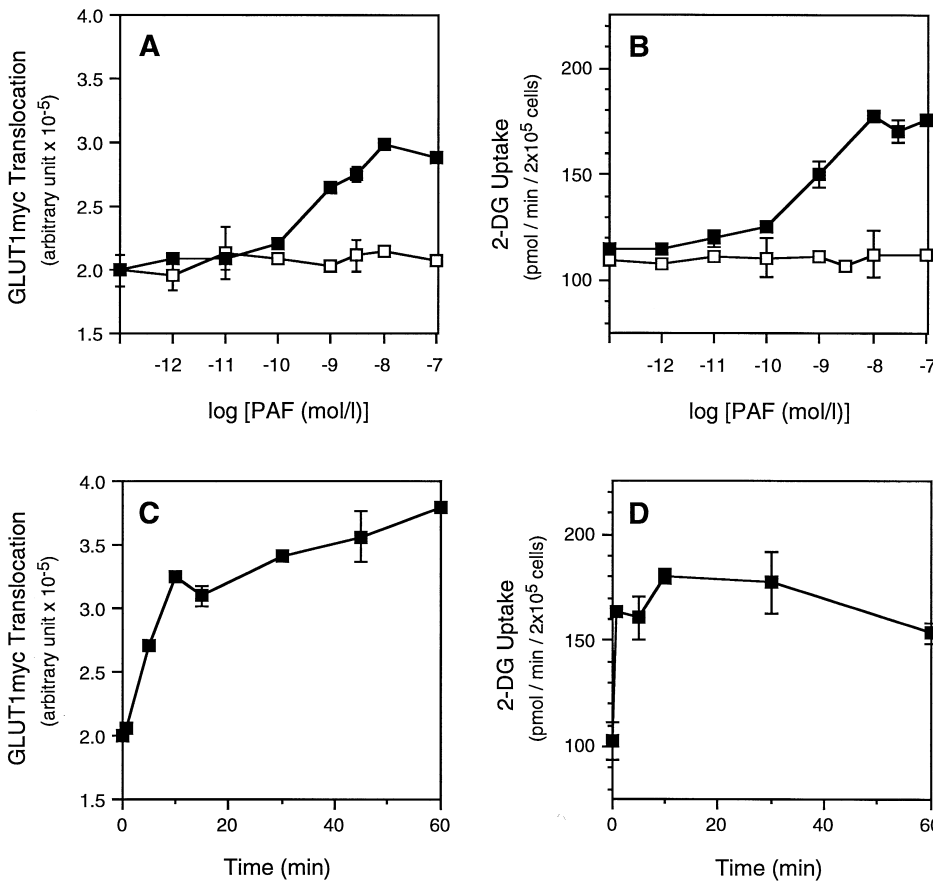


Fig. 4. Dose- and time-dependent GLUT1myc translocation and 2-DG uptake in response to PAF in CHO-GLUT1myc-PAF•R cells. A and B, the parent cells (CHO-GLUT1myc cells) (○) and cells expressing PAF•R (CHO-GLUT1myc-PAF•R cells) (■) were stimulated with various concentrations of PAF for 10 min at 37 °C. The GLUT1myc translocation (A) and 2-DG uptake (B) are shown. C and D, CHO-GLUT1myc-PAF•R cells were stimulated with 1 × 10⁻⁷ mol/l PAF for the indicated periods at 37 °C. The GLUT1myc translocation (C) and 2-DG uptake (D) are shown.

due to the translocation of GLUT1 in CHO cells expressing chemoattractant-receptors.

We reported that phosphatidylinositol 3-kinase (PI 3-kinase) is essential for insulin-triggered GLUT4 translocation (27), and that GLUT4 translocation is triggered not only by insulin but also by activations of G-protein coupled receptors (10, 11). To determine the molecular mechanisms of fMLP- or PAF-induced GLUT1 translocation, effects of IAP (Gi inhibitor) and wortmannin (PI 3-kinase inhibitor) on the chemoattractant-stimulated GLUT1 translocation were examined using CHO cells. The IAP-pretreatment completely inhibited the fMLP-stimulated GLUT1 translocation in CHO-GLUT1myc-fMLP•R cells, but the PAF-stimulated GLUT1 translocation was only slightly inhibited by IAP in CHO-GLUT1myc-PAF•R (Fig. 5). Because the GLUT1 translocation by fMLP is transmitted through Gi coupling to the receptor, and PAF-triggered GLUT1 translocation is mediated through both Gi and Gq coupling to the receptor, IAP is not able to inhibit the PAF-triggered translocation completely (10, 17). Pretreatment of wortmannin at 1×10^{-7} mol/l completely inhibited the insulin-stimulated GLUT1 translocation, but only partly inhibited the fMLP-stimulated GLUT1 translocation (Fig. 5A). The insulin-induced GLUT1 translocation is more sensitive to wortmannin than is the fMLP-induced GLUT1 translocation. The PAF-stimulated GLUT1 translocation was not inhibited by wortmannin (Fig. 5 B). The PAF receptor apparently transmitted the signal for GLUT1 translocation via Gq, which is an insulin-independent and PI 3-kinase-independent pathway (11). However, the pathway of fMLP-stimulated GLUT1 translocation via Gi may be partly mediated through PI 3-kinases (see DISCUSSION). Next, we asked whether serine/threonine protein kinase Akt (also called PKB or RAC-PK) existing downstream of PI 3-kinase would be activated by fMLP- or PAF-treatment. Akt kinase activity is regulated by PI 3-kinase products, phosphatidylinositol 3,4-diphosphate and phosphatidylinositol 3,4,5-triphosphate, and activation of the kinase coincides with phosphorylations at Thr308 and Ser473 by 3-phosphoinositide (PI 3-kinase products)-dependent protein kinase (PDK) 1/2 (28, 29), detectable from the slower migration, compared to non-activated Akt, on western analysis (30). As shown in Fig. 6, fMLP activated Akt kinase in both CHO cells and PMNs, but PAF activated only a very small amount of this kinase. PAF slightly transmitted the signal of Akt phosphorylation via Gi because the PAF receptor is partly coupled to

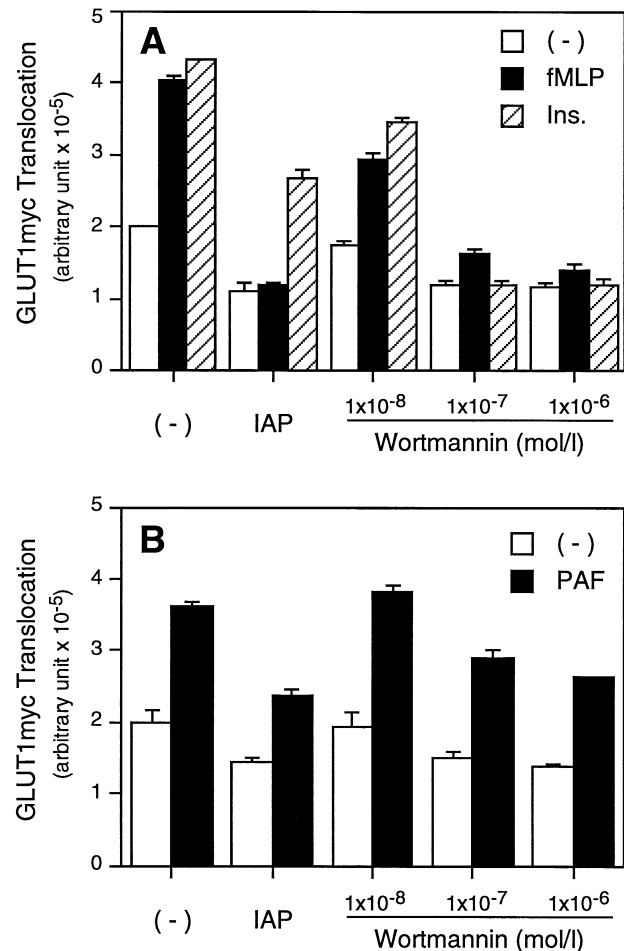


Fig. 5. Effects of IAP and wortmannin on GLUT1myc translocation in response to fMLP or PAF in CHO-GLUT1myc cells expressing its receptor. A, the CHO-GLUT1myc-fMLP•R cells were stimulated with 1×10^{-7} mol/l fMLP, 1×10^{-7} mol/l insulin, or buffer alone after pretreatment with 100 ng/ml IAP or the indicated concentration of wortmannin for 20 min at 37 °C, and GLUT1myc translocation was measured. B, the CHO-GLUT1myc-PAF•R cells were stimulated with 1×10^{-7} mol/l PAF or buffer alone after pretreatment with 100 ng/ml IAP or the indicated concentration of wortmannin for 20 min at 37 °C, and GLUT1myc translocation was measured.

Gi and may be mainly coupled to Gq. While the phosphorylated Akt in CHO cells continued to be activated for 5–10 min, at maximal levels, Akt in phagocytes showed a maximum of activation at 30–90 sec (Fig. 6). The Akt activation process in phagocytes was more transient than that in CHO cells. In phagocytes, the receptor activated by fMLP or PAF triggers activation or phosphorylation of mitogen-activated protein kinase (MAPK) families which include extracellular signal-regulated kinases (ERKs), p38 MAPK, and c-Jun N-terminal kinases (31, 32). We also found that both fMLP and PAF in CHO cells cause phosphorylation of ERKs, using western blots and phospho-specific antibody against ERK (data not shown). The same protein kinases

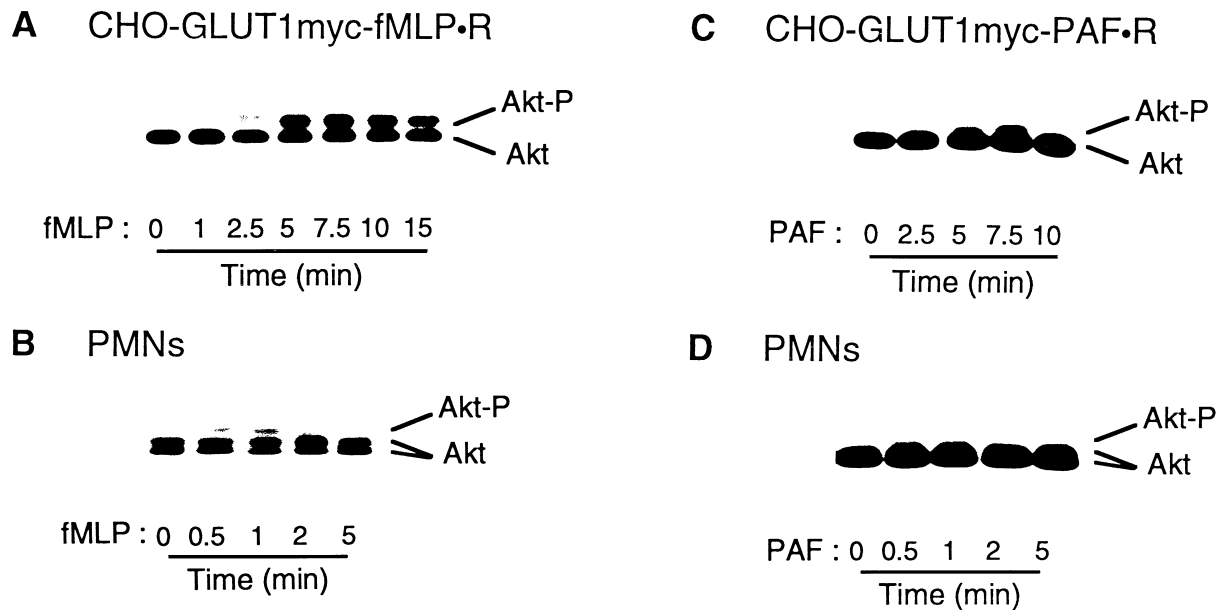


Fig. 6. Time course of Akt kinase activation in the CHO cells and rat PMNs. CHO-GLUT1myc-fMLP•R cells, CHO-GLUT1myc-PAF•R cells, and rat PMNs were stimulated with 1×10^{-7} mol/l fMLP or 1×10^{-7} mol/l PAF for the indicated periods at 37°C . Cell lysates (10 μg) were separated on 7% SDS-PAGE gels and activation of Akt was detected on western blots using an anti-Akt antibody. Akt-P and Akt indicates phosphorylated and non-phosphorylated Akt, respectively.

were activated by fMLP- or PAF-stimulation in phagocytes and in CHO cells.

DISCUSSION

As a first approach to elucidating the mechanism(s) of glucose uptake in phagocytes, investigators used the principles of enzyme kinetic analysis and the Michaelis-Menten equation, similar to a previous study of the mechanism by which insulin increases glucose uptake by adipocytes (33). Several groups of researchers found that the fMLP-induced increase in glucose uptake by PMNs was associated with a decrease in the K_m for this process, but the V_{max} remained unchanged (21, 22, 34). These results indicated that the enhanced glucose uptake in chemoattractant-stimulated PMNs involves a mechanism other than an increase in the number of cell surface transporters. They reported that the rapid glucose uptake on fMLP-stimulation in PMNs was consistent with an increased intrinsic activation of glucose transporter molecules, but the possibility that transporter translocation contributes to the stimulated glucose uptake has to be considered. On the other hand, Okuno and Gliemann reported that both an increase in the V_{max} and a decrease in K_m are important for chemoattractant-stimulated glucose uptake (13). Malide *et al* used confocal laser

scanning microscopy to define the subcellular localization of GLUT1 in cultured macrophages. In response to long term treatment with PMA or fMLP, GLUT1 was translocated to the cell surface (26). However, the molecular mechanism related to the acute regulation of glucose uptake in phagocytes is poorly understood. In the present study we used CHO cells stably expressing both GLUT1myc and chemoattractant-receptors to elucidate the molecular mechanisms of acute regulation of glucose uptake in response to chemoattractants, because of technical difficulties in estimating the amount of endogenous GLUT1 on the cell surface of phagocytes. We found that in CHO cells stimulated with fMLP or PAF, glucose uptake is increased through GLUT1 translocation, and it seemed that the same intracellular events are triggered by fMLP or PAF in phagocytes.

fMLP- and PAF-induced GLUT1 translocation are initiated by ligands binding to the receptors. fMLP receptors couple with the heterotrimeric G-protein, G_i . PAF receptors couple with G_i and G_q . Upon activation of G_q by the binding ligands, phospholipase C (PLC)- β is activated and PLC- β activation results in the generation of two secondary messengers, inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG) (1,2). IP_3 triggers an increase in the cytosolic Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) from intracellular pools, and DAG acts in conjunction with calcium to activate various isoforms of protein kinase C (PKC) (2). The

activated PKC catalyzes various protein phosphorylations that finally result in the activation of various phagocyte functions (2). In PMNs, increased $[Ca^{2+}]_i$ in the presence of fMLP or PAF triggered activation of glycogen phosphorylase activity (data not shown) (3). But Ca^{2+} ionophores did not trigger GLUT1 translocation (10), and phorbol 12,13-dibutyrate (PDBu) pretreatment which induced downregulation of PKC, had practically no effect on fMLP- and PAF-induced GLUT1 translocation in CHO cells (unpublished data). Therefore, fMLP- and PAF-stimulated GLUT1 translocation seems to be mediated by the $G\alpha$ subunit (G_i or G_q) through some unknown pathway(s), following G-protein activation but probably is not a secondary phenomenon as the result of phosphatidylinositol 4,5-diphosphate breakdown.

Tan *et al* reported that a PKC inhibitor, calphostin C, and a tyrosine kinase inhibitor, genistein, inhibited fMLP-stimulated 2-DG uptake in human peripheral blood PMNs (21). In CHO cells expressing the fMLP receptor, calphostin C and genistein at same concentrations also inhibited fMLP-stimulated GLUT1 translocation (unpublished data). Inhibitor studies suggest that the acute activation of glucose transport in response to fMLP involves tyrosine and serine/threonine kinase.

Recently, several investigators suggested that Akt kinase activation is closely related to GLUT4 translocation (35, 36). We also found that G_i -activation but not G_q -activation induced 3-phosphoinositide accumulation via PI 3-kinase activation (unpublished data). Therefore, the mechanism(s) of G_i -triggered GLUT1 translocation by fMLP may involve Akt activation by PDK 1/2 (28, 29). Several isoforms of PI 3-kinase have been characterized in PMNs, for example, the classical type α (p85/p110) and type γ PI 3-kinase (p101/p110), which is activated by $\beta\gamma$ subunits of fMLP-stimulated G-protein, but only PI 3-kinase type α was expressed in CHO cells (37-39). Differences in the expression of the PI 3-kinase subtype and numbers of the receptors may be related to differences in the response time of Akt activation processes between phagocytes and CHO cells. On the other hand, G_q activation did not induce activation of PI 3-kinase, and G_q -triggered GLUT1 translocation by PAF was not related to Akt activation.

The rapid response of glucose uptake to fMLP or PAF in phagocytes is not due to activation of GLUT1 gene expression. Although the possibility that increased transporter affinity for glucose may contribute to the observed increase in glucose

uptake in activated phagocytes cannot be excluded, we propose that phagocytes activated by fMLP or PAF can trigger translocation of GLUT1 from the internal pool to the cell surface.

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