Activators of AMP-activated protein kinase enhance GLUT4 translocation and its glucose transport activity in 3T3-L1 adipocytes

Shinya Yamaguchi,1 Hiroshi Katahira,1 Sachihiko Ozawa,1 Yoko Nakamichi,2 Toshiaki Tanaka,1 Tatsuhiro Shimoyama,1 Kazuto Takahashi,1 Katsuhiko Yoshimoto,1 Mica Ohara Imaizumi,2 Shinya Nagamatsu,2 and Hitoshi Ishida1

1Third Department of Internal Medicine and 2Department of Biochemistry, Kyorin University, Mitaka, Tokyo, Japan

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MATERIALS AND METHODS

Materials. Wortmannin, AICAR, and bovine serum albumin (BSA) were from Sigma (St. Louis, MO), and DNP and 2-deoxy-d-glucose (2-DG) were from Wako (Osaka, Japan). SB-203580 was from Calbiochem (La Jolla, CA). 2-[14C]DG (300 mCi/mmol) was obtained from DuPont-NEN (Boston, MA). Human GLUT4 cDNA was a generous gift from Dr. G. I. Bell (University of Chicago). The GLUT4-eGFP construct was prepared by subcloning the full-length GLUT4 cDNA in frame into the HindII and EcoRI sites of the pcGFP vector (Clontech, Palo Alto, CA) to make a COOH-terminal eGFP fusion.

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Address for reprint requests and other correspondence: H. Ishida, Kyorin Univ. School of Medicine, 6-20-2 Shinkawa, Mitaka, Tokyo 181-8611, Japan (e-mail: ishida@kyorin-u.ac.jp).

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Preparation of cells. 3T3-L1 cells were obtained from the cell bank of Japanese Collection of Research Bioresources (Tokyo, Japan). Cells were seeded and fed every 2–3 days in Dulbecco’s modified Eagle’s medium (DMEM) high glucose supplemented with 50 U/ml penicillin, 50 μg/ml streptomycin, 100 mM MEM sodium pyruvate, and 10% fetal calf serum and were grown under 5% CO₂ at 37°C. At confluence, differentiation was started by addition of medium containing 500 μM isobutylmethylxanthine (IBMX, Sigma), 250 μM dexamethasone (Sigma), and 1.7 μM insulin. After 48 h, this mixture was replaced with fresh medium. Between days 7 and 10 after induction of differentiation, the glucose uptake was determined using 2-[¹⁴C]DG, and the dynamics of GLUT4 translocation were monitored in living cells by transferring the cDNA of GLUT4-eGFP into their nucleus.

Cell microinjection of GLUT4-eGFP cDNA. 3T3-L1 adipocytes were injected with cDNA of GLUT4-eGFP using an Eppendorf microinjector system (Femtojet; Eppendorf, Hamburg, Germany) fit-

Fig. 1. Visualization of GLUT4 translocation using GLUT4-eGFP (enhanced green fluorescent protein) chimeric protein and dynamics of its intracellular distribution after addition of stimulants. Each image was obtained using laser-scanning confocal microscopy before (1), and 4 (2), 10 (3), and 20 min (4) after the following stimulations: A: 10⁻⁷ M insulin alone; B: 10⁻⁷ M insulin + 3 × 10⁻⁸ M wortmannin; C: 10⁻⁷ M insulin + 10⁻⁵ M SB-203580 (an inhibitor of p38 MAPK); D: 10⁻⁴ M 2,4-dinitrophenol (DNP) alone; E: 10⁻⁴ M DNP + 3 × 10⁻⁸ M wortmannin.
ed on to Zeiss Axiovert microscope. In each coverslip, the plasmid of GLUT4-eGFP cDNA adjusted to 50–200 μg/ml in 10 mM Tris·HCl buffer (pH 8.0) plus 1 mM EDTA was injected into the nucleus of ~50 cells. After injection, cells were washed twice with DMEM containing 10% fetal calf serum and incubated for 16–24 h under 5% CO₂ at 37°C. The efficiency of gene transfer into cells was in the range of 8–27%.

Experimental protocol for stimulation with insulin, DNP, or AICAR. After the preincubation of differentiated 3T3-L1 adipocytes in Krebs-Ringer bicarbonate (KRB) buffer containing (in mM) 110 NaCl, 4.4 KCl, 1.45 KH₂PO₄, 1.2 MgCl₂, 2.3 calcium gluconate, 4.8 NaHCO₃, 11 glucose, and 10 HEPES (pH 7.4) and 0.3% BSA (for GLUT4 translocation) or in serum-free Hanks’ buffer (in mM: 136.9 NaCl, 5.6 KCl, 0.34 Na₂HPO₄·7H₂O, 0.44 KH₂PO₄, 1.27 CaCl₂, and 4.2 NaHCO₃ and 0.02% BSA, pH 7.4) (for 2-DG uptake) at 37°C for 140 min, cells were then additionally incubated with 10⁻⁷ M insulin, 10⁻⁴ M DNP or 10⁻³ M AICAR for 20 min. In experiments where the PI3K inhibitor (3 × 10⁻⁸ M wortmannin) or the p38 MAPK inhibitor (10⁻₅ M SB-203085) was used, each agent was added to the buffer 20 min before the addition of insulin, DNP, or AICAR.

Image capture of GLUT4 translocation and its analysis. The transfected 3T3-L1 adipocytes with GLUT4-eGFP were imaged with a laser-scanning confocal microscope LSM 510 (Carl Zeiss, Jena, Germany) to monitor the dynamics of GLUT4 translocation. Transfected cells were on a heated stage adjusted to provide a temperature of 37°C in the bathing KRB buffer. Images were collected using 488-nm excitation wavelength every 1 min. The eGFP signals were analyzed with NIH Image software (version 1.61, National Institute of Mental Health, NIMH Public Inquiries, Bethesda, MD). To quantify the extent of GLUT4-eGFP translocation to the plasma membrane, the ratio of fluorescence intensity in the peripheral region to that in the remaining cellular fluorescence was calculated. The peripheral-to-cellular ratio in the basal state was expressed as 100(%), and the area under the curve during the 20-min stimulation was calculated for the quantitative comparison.

2-DG uptake assay. The differentiated 3T3-L1 adipocytes were serum starved using serum-free Hanks’ buffer (pH 7.4) prior to glucose uptake experiments, as described above, and were preincubated for 140 min. For 2-DG uptake measurements, 0.2 μCi 2-[¹⁴C]DG was then added to the medium containing 1 mM nonra-
dioactive 2-DG. After the incubation for 20 min at room temperature along with each stimulant (insulin, DNP, or AICAR), glucose uptake was stopped by aspiration of the buffer. Cells were rapidly washed several times with 1 ml of ice-cold phosphate-buffered saline and solubilized by the addition of 0.2 M NaOH. Non-specific uptake was determined in parallel in the presence of 10 μM cytochalasin B. The radioactivity associated with the cells was measured using a scintillation counter (LSC-3100; Aloca, Tokyo, Japan), as has been described previously (21).

Statistical analysis. Statistical analysis was performed by analysis of variance using StatView computer software (Abacus, Berkeley, CA). Results are expressed as means ± SE, and P < 0.05 was considered significant.

RESULTS

Visualization of GLUT4 translocation and its dynamics of intracellular distribution. The dynamics of GLUT4-eGFP translocation were visualized and monitored using laser-scanning confocal microscopy. Each image in Fig. 1 indicates the intracellular localization of GLUT4 before (1), and 4 (2), 10 (3), and 20 min (4) after the addition of 10⁻⁷ M insulin, 10⁻⁴ MDNP, or 10⁻³ M AICAR. As indicated in Fig. 1A, insulin stimulation elicited GLUT4 translocation into plasma membrane within 4 min. On the other hand, the time-dependent translocation was almost diminished by the pretreatment with 3 × 10⁻⁸ M wortmannin (Fig. 1B). Administration of DNP or AICAR alone similarly promoted GLUT4 translocation within 4 min in a time-dependent manner (Fig. 1, D and G). The pretreatment with wortmannin, however, failed to inhibit the translocation by DNP or AICAR (Fig. 1, E and H). In addition, the translocation induced by insulin, DNP, or AICAR was not affected by the pretreatment with 10⁻⁵ M SB-203580. (Fig. 1, C, F, and I).

Quantitative assessment of GLUT4 translocation by each stimulant. A significant increase in the peripheral/cellular ratio of GLUT4-eGFP fluorescence intensity was observed 4 min after the addition of insulin (138.2 ± 13.3% of the basal state, P < 0.05), and it consistently continued thereafter (P < 0.05; Fig. 2A). When the cells were pretreated with wortmannin, however, no apparent acceleration of the GLUT4 translocation was found, and the statistically significant suppression was noted in the translocation during the insulin stimulation (P < 0.05; Fig. 2A). DNP and AICAR also significantly enhanced the GLUT4 translocation 4 min after the addition (140 ± 10.5 and 130.7 ± 8.5% of the basal state in DNP and AICAR stimulation, respectively, P < 0.05; Fig. 2, B and C). In contrast, the wortmannin pretreatment failed to suppress the GLUT4 translocation stimulated with DNP or AICAR (Fig. 2, B and C). The addition of SB-203580 did not suppress the translocation by insulin, DNP or AICAR (Fig. 2, A–C). When the area under the curve of peripheral/cellular ratio of fluorescence intensity was calculated (Fig. 3A), it was confirmed that wortmannin could almost completely inhibit the insulin-induced GLUT4 translocation (63.081 ± 8.516 and 181 ± 844 arbitrary units in insulin alone and insulin plus wortmannin, respectively, P < 0.01), but not by DNP or AICAR (Fig. 3, B and C). No significant effect on the accelerated translocation by insulin, DNP, or AICAR was observed by the addition of SB-203580 (Fig. 3, A–C).

Insulin-, DNP-, and AICAR-induced 2-DG uptake inhibition by wortmannin or SB-203580. The stimulation by insulin, DNP, and AICAR enhanced 2-DG uptake 2.8 ± 0.14-, 2.4 ± 0.21-, and 1.7 ± 0.14-fold, respectively (P < 0.01). The addition of wortmannin did not affect the basal 2-DG uptake but markedly suppressed the insulin-induced glucose uptake (5,215 ± 429 and 1,618 ± 277 cpm/10⁶ cells in insulin alone and insulin plus wortmannin, respectively, P < 0.01). Inter-
ently, wortmannin treatment could also significantly inhibit 2-DG uptake by DNP and AICAR (4,625 ± 555 and 2,903 ± 65 cpm/10^6 cells in DNP alone and DNP plus wortmannin, respectively, P < 0.05, and 3,140 ± 381 and 1,891 ± 150 cpm/10^6 cells in AICAR alone and AICAR plus wortmannin, respectively, P < 0.05; Fig. 4A), whereas wortmannin failed to suppress the GLUT4 translocation stimulated by these agents, as has been described above. In another series of experiments, SB-203580 pretreatment was found to significantly inhibit the glucose uptake enhanced by insulin (5,752 ± 190 and 3,692 ± 67 cpm/10^6 cells in insulin alone and insulin plus SB-203580, respectively, P < 0.05) and also by DNP and AICAR (4,062 ± 212 and 3,072 ± 168 cpm/10^6 cells in DNP alone and DNP plus SB-203580, respectively, P < 0.05; 4,880 ± 316 and 2,444 ± 99 cpm/10^6 cells in AICAR alone and AICAR plus SB-203580, respectively, P < 0.05; Fig. 4B).

![Graph A](image1)

![Graph B](image2)

**Fig. 3.** Calculation of area under the curve (AUC) of %peripheral/cellular ratio of GLUT4-eGFP fluorescence intensity after addition of 10^{-7} M insulin (A), 10^{-4} M DNP (B), and 10^{-3} M AICAR (C). Accelerated GLUT4 translocation by insulin was significantly suppressed by 3 × 10^{-8} M wortmannin (P < 0.01) but not by 10^{-3} M SB-203580. DNP- or AICAR-induced GLUT4 translocation was not affected by these agents. N.S., not significant. Data are means ± SE of 6 determinations on different 3T3-L1 cells.

![Graph C](image3)

**Fig. 4.** Stimulation of glucose transport activity in 3T3-L1 adipocytes by insulin, DNP, and AICAR and its suppression by wortmannin (A) and SB-203580 (B). A: addition of wortmannin significantly reduced the glucose uptake stimulated by insulin, as well as that evoked by DNP and AICAR (P < 0.05–0.01). B: enhanced glucose uptakes by insulin, DNP, and AICAR were all significantly suppressed by addition of SB-203580 (P < 0.05). Data are means ± SE of 6 determinations.
DISCUSSION

Through the monitoring system of visualized GLUT4-eGFP translocation (22, 25), we have demonstrated here that the stimulation with DNP and AICAR, known activators of AMPK, promptly promotes the GLUT4 translocation within 4 min, as has been previously reported in the case of insulin (13, 16, 23). In addition, the translocation induced by DNP and AICAR was not affected by wortmannin, suggesting that AMPK-stimulated GLUT4 translocation in 3T3-L1 adipocytes is mediated through the insulin-signaling pathway distal to the site of activated PI3K or through a signaling system distinct from that of insulin. This finding seems to be consistent with the case of enhanced GLUT4 translocation in skeletal muscles, in which AMPK activity has reportedly been elevated through their contraction (11, 17). Because the GLUT4 translocation induced by insulin stimulation and AMPK activation in 3T3-L1 adipocytes was not found to be affected by SB-203580, a known inhibitor of p38 MAPK, the recruitment of GLUT4 to the plasma membrane was unlikely to have been mediated by p38 MAPK signaling.

The addition of AMPK activators AICAR and DNP enhanced glucose uptake in 3T3-L1 adipocytes, as was found in the case of insulin stimulation. In contrast to the case of GLUT4 translocation, however, the glucose transport activity was significantly reduced by the addition of wortmannin. It has been supposed that the magnitude of glucose transport after insulin stimulation is regulated by promoting the GLUT4 translocation via PI3K activation (7, 14, 24) and also through increasing the intrinsic transport activity of GLUT4 by protein phosphorylation via p38 MAPK activation (3, 28, 29). Although wortmannin has been used as an inhibitor of PI3K, this agent has been additionally reported to inhibit insulin-dependent p38α and p38β MAPK activities with an IC_{50} of 6 × 10^{-9} M and 2 × 10^{-9} M, respectively (28). Because the concentration of wortmannin used in this experiment was 3 × 10^{-8} M, it can be expected that it suppresses the p38 MAPK activities in 3T3-L1 adipocytes. In fact, the AMPK-activated glucose transport activity was found to be significantly suppressed by SB-203580 in the present study. Because the magnitude of the 2-DG transport response by insulin and AMPK activators was found to be relatively poor compared with that in previous studies (3, 29), the degree of cell differentiation of 3T3-L1 adipocytes might have been lower in our cell preparations. However, the significance in the reduction of glucose uptake by the addition of wortmannin and SB-203580 could still be valid, because apparent GLUT4 translocation into the plasma membrane of 3T3-L1 adipocytes was observed in the cell preparations similar to those in the glucose uptake assay. It could, accordingly, be speculated that the enhancement of glucose transport induced by AMPK activation in 3T3-L1 adipocytes is mediated by the increase of intrinsic activity through p38 MAPK activation of translocated GLUT4 protein, as has been suggested in the case of skeletal muscle (18). A detailed evaluation would be needed in the future regarding which isoforms of p38α and p38β MAPK can be phosphorylated and activated during this process. However, because it has been demonstrated that SB-203580 inhibited the nucleotide transport system and therefore can indirectly reduce AICAR-stimulated AMPK activity (8), further examinations should be carefully conducted on the exact role of AMPK and p38 MAPK activation in the accelerated glucose uptake activity of GLUT4 protein in 3T3-L1 adipocytes. It has been recently reported that AICAR-stimulated glucose transport was dependent on activation of the extracellular signal-regulated kinase pathway, phospholipase D, and atypical protein kinase C isoforms in myocytes (5). Interestingly, the possible involvement of AMPK-independent signaling has been also proposed, using the system of overexpression of a dominant negative AMPK mutant (26). It should be an interesting issue to be resolved, therefore, how AMPK-dependent and -independent pathways are related to the AMPK-induced glucose uptake observed in 3T3-L1 adipocytes. On the other hand, in adipose cells including 3T3-L1, it has been observed that GLUT1, another isoform of glucose transporters, is also functionally expressed (12). AICAR has been reported to increase the intrinsic activity of GLUT1 protein via activated p38 MAPK to enhance the glucose transport into Clone 9 cells, a rat liver-derived, non-transformed cell line that expresses only the GLUT1 (1, 31). The increased intrinsic activity of GLUT1 as well as GLUT4 could potentially induce the enhancement of glucose uptake after AMPK activation in 3T3-L1 adipocytes. In contrast to our findings, Salt et al. (27) have demonstrated that AICAR does not significantly enhance the translocation of GLUT4 or GLUT1 to the plasma membrane, whereas a modest stimulation of glucose uptake was simultaneously observed. The precise reason for the discrepancy in the translocation of GLUT4 between their results and ours is still unclear at present, but the effect on 3T3-L1 cells might have been too small in their experiments to be detected by the plasma membrane lawn assay, because their method is supposedly less sensitive for the detection compared with ours using the system of the GLUT4-eGFP chimeric protein. Further studies concerning the intracellular system for translocation and activation of various GLUT isoforms are necessary to clarify this issue.

In the present study, it is revealed that AMPK activators AICAR and DNP can accelerate GLUT4 translocation in 3T3-L1 adipocytes, as has been observed in skeletal muscles. The mode of action on the translocation through AMPK activation was as quick, within 4 min, as in the case of insulin stimulation, but it was shown to be mediated by a signaling system distinct from that activated by insulin. On the other hand, the enhancement of glucose uptake was further mediated by the increased intrinsic activity of GLUT4 protein, possibly through p38 MAPK activation. The activation of AMPK in adipocytes would be a new therapeutic target for the glycemic control of type 2 diabetes.

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AMPK-INDUCED GLUT4 TRANSLOCATION


