

# The Antihyperglycemic Drug $\alpha$ -Lipoic Acid Stimulates Glucose Uptake via Both GLUT4 Translocation and GLUT4 Activation

## Potential Role of p38 Mitogen-Activated Protein Kinase in GLUT4 Activation

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The cofactor of mitochondrial dehydrogenase complexes and potent antioxidant  $\alpha$ -lipoic acid has been shown to lower blood glucose in diabetic animals.  $\alpha$ -Lipoic acid enhances glucose uptake and GLUT1 and GLUT4 translocation in 3T3-L1 adipocytes and L6 myotubes, mimicking insulin action. In both cell types, insulin-stimulated glucose uptake is reduced by inhibitors of p38 mitogen-activated protein kinase (MAPK). Here we explore the effect of  $\alpha$ -lipoic acid on p38 MAPK, phosphatidylinositol (PI) 3-kinase, and Akt1 in L6 myotubes.  $\alpha$ -Lipoic acid (2.5 mmol/l) increased PI 3-kinase activity (31-fold) and Akt1 (4.9-fold). Both activities were inhibited by 100 nmol/l wortmannin.  $\alpha$ -Lipoic acid also stimulated p38 MAPK phosphorylation by twofold within 10 min. The phosphorylation persisted for at least 30 min. Like insulin,  $\alpha$ -lipoic acid increased the kinase activity of the  $\alpha$  (2.8-fold) and  $\beta$  (2.1-fold) isoforms of p38 MAPK, measured by an *in vitro* kinase assay. Treating cells with 10  $\mu$ mol/l of the p38 MAPK inhibitors SB202190 or SB203580 reduced the  $\alpha$ -lipoic acid-induced stimulation of glucose uptake by 66 and 55%, respectively. In contrast, SB202474, a structural analog that does not inhibit p38 MAPK, was without effect on glucose uptake. In contrast to 2-deoxyglucose uptake, translocation of GLUT4myc to the cell surface by either  $\alpha$ -lipoic acid or insulin was unaffected by 20  $\mu$ mol/l of SB202190 or SB203580. The results suggest that inhibition of 2-deoxyglucose uptake in response to  $\alpha$ -lipoic acid by inhibitors of p38 MAPK is independent of an effect on GLUT4 translocation. Instead, it is likely that regulation of transporter activity is sensitive to these inhibitors. *Diabetes* 50:1464–1471, 2001

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ANOVA, analysis of variance; ATF, activating transcription factor; DTT, dithiothreitol; IRS-1, insulin receptor substrate-1; MAPK, mitogen-activated protein kinase; MEM, minimum essential medium; PI, phosphatidylinositol.

$\alpha$ -Lipoic acid (also known as thioctic acid) is a potent biological antioxidant and a naturally occurring cofactor of mitochondrial dehydrogenase complexes. In clinical trials,  $\alpha$ -lipoic acid improved glucose metabolism in patients with type 2 diabetes (1,2). Furthermore, this compound has proven beneficial in the treatment of diabetic neuropathy (3,4). In animal models,  $\alpha$ -lipoic acid restored insulin-stimulated glucose uptake into insulin-resistant skeletal muscle of obese Zucker rats (5–7). Treatment of streptozotocin-induced diabetic rats with  $\alpha$ -lipoic acid caused a significant reduction in plasma glucose levels and enhanced insulin-stimulated glucose uptake into muscle (8). We have shown that  $\alpha$ -lipoic acid stimulates glucose uptake in 3T3-L1 adipocytes by rapid translocation of the glucose transporters GLUT1 and -4 from an internal membrane fraction to the plasma membrane (9). Furthermore, treatment of 3T3-L1 adipocytes with  $\alpha$ -lipoic acid caused increased tyrosine phosphorylation of the insulin receptor substrate-1 (IRS-1) and activation of both phosphatidylinositol (PI) 3-kinase and the serine/threonine kinase Akt1 (10). In this way,  $\alpha$ -lipoic acid differs from other stimuli that increase glucose uptake, such as contraction and hypoxia, which stimulate glucose uptake by a PI 3-kinase-independent mechanism (11,12).  $\alpha$ -Lipoic acid is also unique among current antihyperglycemic therapies in its direct engagement of the insulin signaling pathway.

It is now apparent that increased plasma membrane glucose transporter content is insufficient to fully account for the insulin-stimulated elevation in glucose uptake (13). It has been proposed that insulin-stimulated glucose transport might include changes in the intrinsic activity of GLUTs (14). Similarly, in cells in culture, glucose uptake can be stimulated by protein synthesis inhibitors, such as anisomycin, without any increase in cell surface glucose transporters (15,16). In addition, there are reports that glucose uptake can be reduced despite normal GLUT4 translocation (17,18). These studies suggest that the intrinsic activity of cell surface glucose transporters may be regulated by insulin and other agents that stimulate glucose uptake.

Whether  $\alpha$ -lipoic acid is able to modulate the intrinsic activity of cell-surface glucose transporters is unknown. We have recently shown that four inhibitors of p38 mitogen-activated protein kinase (MAPK) reduce insulin-stimulated glucose uptake without altering the translocation of glucose transporters (18,19). These results suggest that p38 MAPK may contribute to the regulation of the intrinsic activity of glucose transporters by insulin.

In the present study, we examined the effect of  $\alpha$ -lipoic acid and insulin on the phosphorylation and activation of different isoforms of p38 MAPK in L6 GLUT4myc myotubes. In addition, we explore the effect of two selective p38 MAPK inhibitors, SB202190 and SB203580, on the stimulation of glucose uptake and GLUT4myc translocation. The results support the hypothesis that stimulation of glucose transport consists of at least two contributory mechanisms: translocation of GLUT4 to the plasma membrane and stimulation of their intrinsic activity.

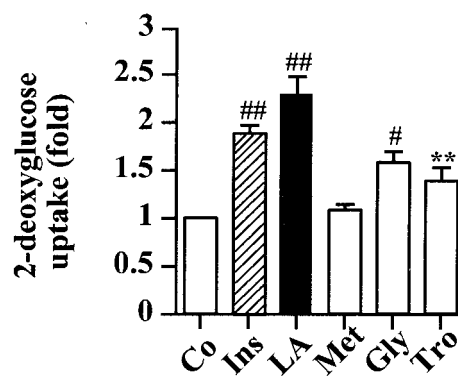
## RESEARCH DESIGN AND METHODS

**Materials.** R(+)  $\alpha$ -lipoic acid was obtained from ASTA Medica (Frankfurt, Germany). Metformin and glyburide (glybenclamide) were purchased from Sigma (St. Louis, MO). Troglitazone was a gift from Dr. A. Saltiel (Parke-Davis Pharmaceutical Research, Ann Arbor, MI). Human insulin (Humulin R) was obtained from Eli Lilly Canada (Toronto, ON, Canada). The pyrimidinyl imidazoles SB202190, SB202474, and SB203580 were purchased from Calbiochem (La Jolla, CA). Polyclonal antibodies to p38 MAPK $\alpha$ , p38 MAPK $\beta$ , Akt1 (C20), and monoclonal anti-myc antibody (9E10) were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho-p38 MAPK antibody was from New England Biolabs (Beverly, MA). Anti-IRS-1 antibody and Akt substrate peptide (Crosstide) were from Upstate Biotechnology (Lake Placid, NY). Activating transcription factor (ATF)-2 fusion protein was purchased from New England Biolabs (Beverly, MA). *O*-Phenylethylamine dihydrochloride and wortmannin were from Sigma. L6 GLUT4myc myoblasts were differentiated into myotubes as described previously (20). Before all experimental manipulations, L6 GLUT4myc myotubes were deprived of serum for 4 h.

**Determination of 2-deoxy- $^3$ H-D-glucose uptake.** 2-Deoxyglucose uptake was measured as described earlier (18). Glyburide and troglitazone stock solutions were prepared using DMSO and subsequently diluted with  $\alpha$ -minimum essential medium (MEM) before application to the cells. Metformin was dissolved in  $\alpha$ -MEM.

**p38 MAPK and Akt1 phosphorylation.** Phosphorylation of p38 MAPK and Akt1 was detected essentially as described earlier (21). Briefly, after incubating cells with the indicated agent, they were lysed with 150  $\mu$ l concentrated 2 $\times$  Laemmli sample buffer supplemented with 1 mmol/l dithiothreitol (DTT), 1 mmol/l  $\text{Na}_3\text{VO}_4$ , 100 nmol/l okadaic acid, protease inhibitors (1 mmol/l benzamide, 10  $\mu$ mol/l E-64, 1  $\mu$ mol/l leupeptin, 1  $\mu$ mol/l pepstatin A, and 0.2 mmol/l phenylmethylsulfonyl fluoride), and 7.5%  $\beta$ -mercaptoethanol. The lysates were transferred to Eppendorf tubes, vortexed for 1 min, passed five times through a 25-gauge syringe, and heated for 15 min at 65°C. Samples were centrifuged for 5 min (1,000 rpm) then 40  $\mu$ l (50  $\mu$ g protein) of the supernatant was resolved by 10% SDS-PAGE, electrotransferred onto polyvinylidene fluoride membranes, and immunoblotted for phospho-p38 MAPK or for phospho-T308 and phospho-S473 Akt1 (1:1,000 dilution of primary antibodies).

**In vitro p38 MAPK activity assay.** Protein kinase activity was measured as described (18). Anti-p38 MAPK $\alpha$  or anti-p38 MAPK $\beta$  antibodies (2  $\mu$ g per condition) were preadsorbed to protein A or protein G Sepharose beads, respectively, by incubating overnight at 4°C under constant rotation. Beads were washed twice with 1 ml cold phosphate-buffered saline, once with 600  $\mu$ l cold lysis buffer (containing 50 mmol/l HEPES, pH 7.6, 150 mmol/l NaCl, 10% glycerol [vol/vol], 1% Triton X-100 [vol/vol], 30 mmol/l sodium pyrophosphate, 10 mmol/l NaF, and 1 mmol/l EDTA), and were resuspended in lysis buffer. L6 GLUT4myc myotubes were incubated either with 100 nmol/l insulin for 10 min or 2.5 mmol/l  $\alpha$ -lipoic acid for 15 min. Thereafter, cells were lysed in 1 ml lysis buffer supplemented with protease inhibitors (see above), 1 mmol/l  $\text{Na}_3\text{VO}_4$ , 1 mmol/l DTT, and 100 nmol/l okadaic acid. The p38 MAPK isoforms were immunoprecipitated for 2–3 h with the preadsorbed Sepharose beads. Immunocomplexes were isolated and washed four times as described (18). Immunocomplexes were then incubated for 30 min at 30°C with 50  $\mu$ l reaction mixture (kinase buffer containing 5  $\mu$ mol/l ATP, 2  $\mu$ Ci [ $^{32}$ P]ATP, and 2  $\mu$ g ATF-2 fusion protein per condition). Thereafter, the samples were spotted on phosphocellulose paper (Whatman P81), washed four times with phosphoric



**FIG. 1.**  $\alpha$ -Lipoic acid stimulates glucose transport more effectively than glyburide or troglitazone in L6 myotubes. Cells were preincubated for 30 min with insulin (100 nmol/l),  $\alpha$ -lipoic acid (2.5 mmol/l), metformin (2 mmol/l), glyburide (0.5 mmol/l), or troglitazone (11  $\mu$ mol/l). 2-Deoxyglucose uptake was determined over a 5-min period. Results are the mean  $\pm$  SE of six independent experiments. \*\* $P$  < 0.01, # $P$  < 0.001, and ## $P$  < 0.0001 compared with control (CO).

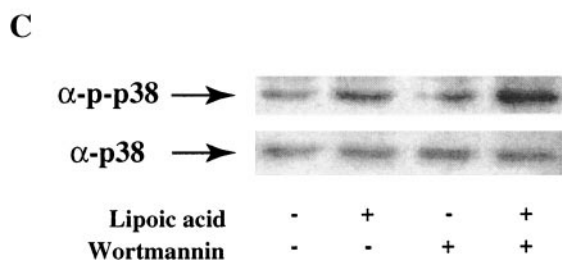
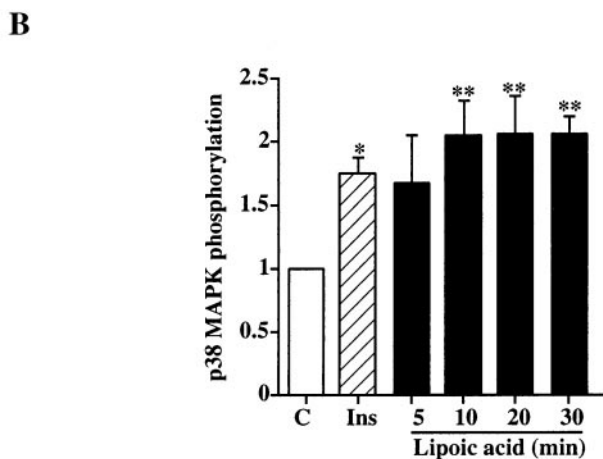
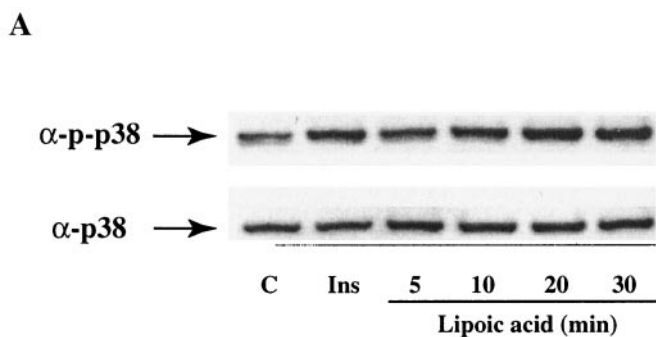
acid (0.125 mol/l) and once with distilled water, and then subjected to liquid-scintillation counting.

**Other assays and data analysis.** Detection of myc-tagged GLUT4 at the cell surface was measured by an antibody-coupled colometric assay essentially as described previously (20). PI 3-kinase activity associated with IRS-1 immunoprecipitates, and Akt1 kinase assay was performed as reported earlier (22). Statistical analysis was performed using the analysis of variance test (ANOVA and Fisher's multiple comparisons test or with the Student's *t* test, as indicated in RESULTS.

## RESULTS

**Action of several antihyperglycemic agents on 2-deoxyglucose uptake in L6 myotubes.** To begin this study, we compared the ability of several currently used antidiabetic agents vis-à-vis insulin and  $\alpha$ -lipoic acid in their ability to stimulate glucose uptake in L6 myotubes overexpressing GLUT4myc. Previous studies have shown that sulfonylureas (e.g., glyburide), biguanides (e.g., metformin), and thiazolidinediones (e.g., troglitazone) stimulate glucose uptake into muscle cells in culture only after prolonged incubation, i.e., hours to days (23–25). Figure 1 shows that  $\alpha$ -lipoic acid effectively stimulated 2-deoxyglucose uptake into L6 myotubes within 30 min (by  $2.3 \pm 0.2$ -fold over basal,  $P$  < 0.0001) to a level comparable with that elicited by insulin. In contrast, glyburide and troglitazone led to a much smaller increase in 2-deoxyglucose uptake ( $1.55 \pm 0.15$ -fold,  $P$  < 0.001, and  $1.4 \pm 0.1$ -fold,  $P$  < 0.01, respectively), and metformin did not stimulate glucose uptake ( $1.1 \pm 0.05$ -fold,  $P$  = 0.57) in the time period studied. These results highlight the difference in action of the various antihyperglycemic agents, and they underscore the importance of understanding the mechanism of action of  $\alpha$ -lipoic acid, the agent more closely mimicking the insulin effect.

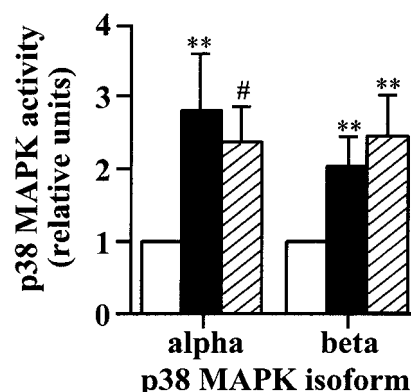
**$\alpha$ -Lipoic acid stimulates p38 MAPK phosphorylation and kinase activity.** Activation of p38 MAPK requires phosphorylation on tyrosine and threonine residues. We have recently shown that insulin increased the phosphorylation of p38 MAPK in rat skeletal muscle (21) and 3T3-L1 adipocytes (18). It is unknown if insulin-mimetic agents, such as  $\alpha$ -lipoic acid, also cause an increase in p38 MAPK phosphorylation. Figure 2A and B shows that  $\alpha$ -lipoic acid leads to a rapid phosphorylation of p38 MAPK with maximum stimulation ( $2.0 \pm 0.3$ -fold,  $P$  < 0.01, ANOVA) ob-



**FIG. 2.** Time-dependent stimulation of p38 MAPK phosphorylation by  $\alpha$ -lipoic acid. L6 GLUT4myc myotubes were treated for the indicated time periods with 2.5 mmol/l  $\alpha$ -lipoic acid or for 10 min with 100 nmol/l insulin. Lysates (50  $\mu$ g) were resolved by 10% SDS-PAGE and immunoblotted with anti-phospho-specific p38 MAPK antibody. **A:** Representative immunoblot (upper immunoblot). The immunoblot shown was stripped of bound antibodies and then reprobed for p38 MAPK. **B:** Immunoblots were scanned within the linear range and quantitated using the computer software National Institutes of Health Image. The quantitated values represent the mean  $\pm$  SE of five experiments. All values are expressed relative to control p38 MAPK phosphorylation, which was assigned a value of 1. \* $P < 0.05$ , \*\* $P < 0.01$  compared with control. **C:** L6 GLUT4myc myotubes were treated for 15 min with or without 2.5 mmol/l  $\alpha$ -lipoic acid after a preincubation with 100 nmol/l wortmannin for 20 min. Lysates (50  $\mu$ g) were resolved by 10% SDS-PAGE and immunoblotted with anti-phospho-specific p38 MAPK antibody. A representative immunoblot of four independent experiments is shown (upper immunoblot). The immunoblot was stripped of bound antibodies and then reprobed for p38 MAPK (bottom immunoblot) to ascertain equal sample loading.

served at 10 min. p38 MAPK phosphorylation remained elevated for at least 30 min. The degree of phosphorylation was comparable with that elicited by insulin. Preincubation with 100 nmol/l wortmannin for 20 min did not inhibit  $\alpha$ -lipoic acid-stimulated p38 MAPK phosphorylation (Fig. 2C).

Four isoforms of p38 MAPK have been described to date



**FIG. 3.** Activation of p38 MAPK  $\alpha$  and  $\beta$  isoforms by  $\alpha$ -lipoic acid. Cells were treated for 15 min with 2.5 mmol/l  $\alpha$ -lipoic acid or for 10 min with 100 nmol/l insulin. p38 MAPK  $\alpha$  or  $\beta$  were immunoprecipitated, and kinase activity was determined by an in vitro kinase assay using ATF-2 as substrate. Results are the mean  $\pm$  SE of five experiments. Nonspecific activity was determined using an irrelevant IgG and was subtracted. Basal kinase activity was assigned a value of 1 and insulin- or  $\alpha$ -lipoic acid-stimulated activity is expressed relative to this value.  $\square$ , basal;  $\blacksquare$ ,  $\alpha$ -lipoic acid;  $\hatched$  insulin. \*\* $P < 0.01$ , # $P < 0.001$  compared with basal.

(26), and it is not known which isoforms are activated by insulin in cells in culture. We have previously shown that insulin activates p38 MAPK  $\alpha$  and  $\beta$  isoforms in rat skeletal muscle. Here we examined which p38 MAPK isoforms are activated by  $\alpha$ -lipoic acid and insulin in L6 muscle cells in culture. p38 MAPK  $\alpha$  and  $\beta$  isoforms were immunoprecipitated, and kinase activity was measured by an in vitro kinase assay (27,28). The results shown in Fig. 3 demonstrate that  $\alpha$ -lipoic acid (15 min) caused a  $2.8 \pm 0.8$ -fold increase in p38 MAPK  $\alpha$  activity ( $P < 0.01$ , Z test) and a  $2.1 \pm 0.4$ -fold increase in p38 MAPK  $\beta$  activity ( $P < 0.01$ ; Z test). Similarly, insulin caused a  $2.4 \pm 0.5$ -fold increase in p38 MAPK  $\alpha$  activity and a  $2.5 \pm 0.6$ -fold increase in p38 MAPK  $\beta$  activity.

**SB202190 and SB203580 reduce  $\alpha$ -lipoic acid-stimulated 2-deoxyglucose uptake.** In Figs. 2 and 3, we showed that  $\alpha$ -lipoic acid and insulin activated p38 MAPK. We have previously demonstrated that inhibition of p38 MAPK with SB203580 reduced insulin-stimulated glucose uptake in 3T3-L1 adipocytes and L6 myotubes (18). Here we determined the effect of inhibition of p38 MAPK using SB202190 and SB203580 on  $\alpha$ -lipoic acid-stimulated glucose uptake. Results illustrated in Fig. 4 show that treatment with  $\alpha$ -lipoic acid caused a  $2.0 \pm 0.1$ -fold increase in 2-deoxyglucose uptake (control  $8.4 \pm 1.2$  pmol  $\cdot$  min $^{-1}$   $\cdot$  mg $^{-1}$  protein,  $\alpha$ -lipoic acid  $16.4 \pm 2.0$  pmol  $\cdot$  min $^{-1}$   $\cdot$  mg $^{-1}$ ,  $P < 0.001$ , ANOVA). Similarly, insulin (100 nmol/l, 30 min) caused a  $2.0 \pm 0.1$ -fold increase in 2-deoxyglucose uptake (insulin  $16.6 \pm 1.8$  pmol  $\cdot$  min $^{-1}$   $\cdot$  mg $^{-1}$ ,  $P < 0.001$ , ANOVA). Pretreatment of cells for 20 min with 10  $\mu$ mol/l SB202190 (Fig. 4A) reduced  $\alpha$ -lipoic acid-stimulated 2-deoxyglucose uptake by  $\sim 66\%$  (SB202190  $6.8 \pm 1.0$  pmol  $\cdot$  min $^{-1}$   $\cdot$  mg $^{-1}$ , SB202190 +  $\alpha$ -lipoic acid  $9.6 \pm 0.4$  pmol  $\cdot$  min $^{-1}$   $\cdot$  mg $^{-1}$ ,  $P < 0.001$ , ANOVA). The same concentration of SB202190 reduced insulin-stimulated 2-deoxyglucose uptake by  $\sim 67\%$  (insulin + SB202190  $9.3 \pm 0.9$  pmol  $\cdot$  min $^{-1}$   $\cdot$  mg $^{-1}$ ,  $P < 0.01$ , ANOVA). Pretreatment of cells with 10  $\mu$ mol/l SB203580 (Fig. 4B) reduced  $\alpha$ -lipoic acid-stimulated 2-deoxyglucose uptake by  $\sim 55\%$  (SB203580  $6.6 \pm 0.9$  pmol  $\cdot$  min $^{-1}$   $\cdot$  mg $^{-1}$ ,  $\alpha$ -lipoic acid + SB203580  $10.3 \pm 1.2$  pmol  $\cdot$  min $^{-1}$   $\cdot$  mg $^{-1}$ ,  $P < 0.01$ , ANOVA). Like-

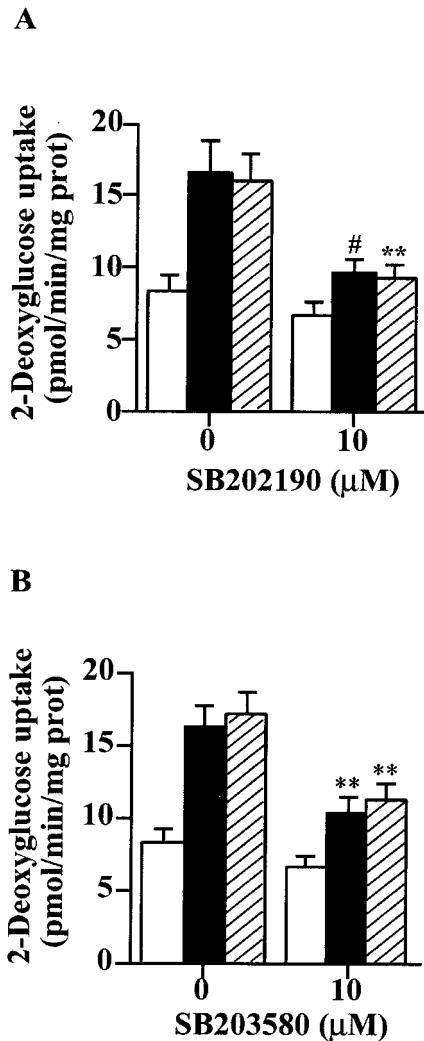


FIG. 4.  $\alpha$ -Lipoic acid-stimulated glucose uptake is inhibited by the p38 MAPK inhibitors SB202190 and SB203580. Cells were incubated for 20 min with or without 10  $\mu$ mol/l of SB202190 (A) and SB203580 (B) before stimulation with 2.5 mmol/l  $\alpha$ -lipoic acid or 100 nmol/l insulin for 30 min. 2-Deoxyglucose uptake was determined over a 5-min period. Results are the mean  $\pm$  SE of six independent experiments. <sup>\*</sup> $P < 0.01$ , <sup>\*\*</sup> $P < 0.001$  compared with the respective control. □, basal; ■,  $\alpha$ -lipoic acid; ▨, insulin.

wise, insulin-stimulated 2-deoxyglucose uptake was reduced by 47% (insulin + SB203580  $11.3 \pm 1.1$  pmol  $\cdot$  min<sup>-1</sup>  $\cdot$  mg<sup>-1</sup>,  $P < 0.01$ , ANOVA). Higher concentrations of SB202190 or SB203580 (up to 20  $\mu$ mol/l) did not reduce the insulin- or  $\alpha$ -lipoic acid-stimulated 2-deoxyglucose uptake further (data not shown). Neither inhibitor caused any significant reduction in the basal rate of 2-deoxyglucose uptake (control vs. SB202190,  $P = 0.40$ , ANOVA; control vs. SB203580,  $P = 0.31$ , ANOVA).

To explore if the effect obtained with SB202190 and SB203580 on  $\alpha$ -lipoic acid-stimulated 2-deoxyglucose uptake was specific to these inhibitors, we used the structural analog SB202474, which is ineffective toward p38 MAPK (18). This compound had no significant effect on 2-deoxyglucose uptake stimulated by either  $\alpha$ -lipoic acid ( $\alpha$ -lipoic acid  $11.4 \pm 1.4$  pmol  $\cdot$  min<sup>-1</sup>  $\cdot$  mg<sup>-1</sup>,  $\alpha$ -lipoic acid + SB202474  $10.5 \pm 1.0$  pmol  $\cdot$  min<sup>-1</sup>  $\cdot$  mg<sup>-1</sup>,  $P = 0.5$ , ANOVA) or insulin (insulin  $10.6 \pm 0.9$  pmol  $\cdot$  min<sup>-1</sup>  $\cdot$  mg<sup>-1</sup>, insulin + SB202474  $9.4 \pm 0.7$  pmol  $\cdot$  min<sup>-1</sup>  $\cdot$  mg<sup>-1</sup>,  $P = 0.33$ , ANOVA).

$\alpha$ -Lipoic acid-stimulated GLUT4 translocation is blocked by wortmannin but is not reduced by SB202190 or SB203580. To determine whether inhibition of glucose

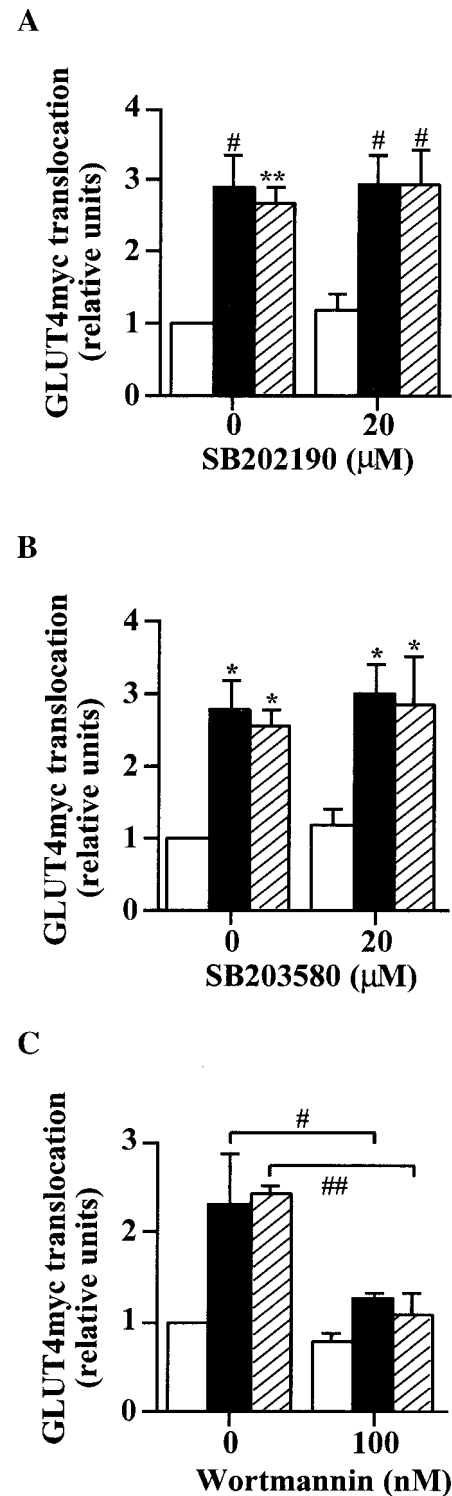
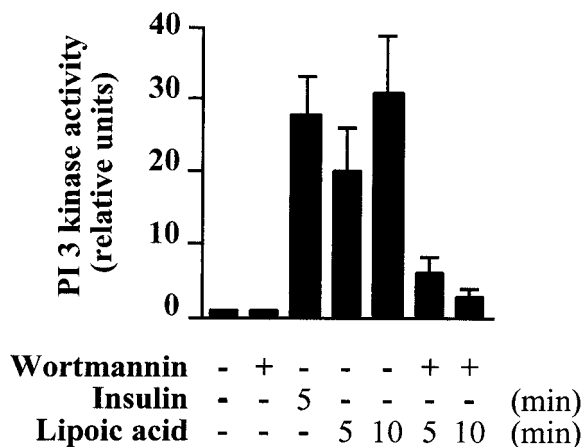


FIG. 5.  $\alpha$ -Lipoic acid-stimulated GLUT4 translocation is inhibited by wortmannin but not by the p38 MAPK inhibitors SB202190 and SB203580. L6 GLUT4myc myotubes were incubated for 20 min with or without 20  $\mu$ mol/l of SB202190 (A), SB203580 (B), or 100 nmol/l wortmannin (C) before stimulation with 2.5 mmol/l  $\alpha$ -lipoic acid or 100 nmol/l insulin for 30 min. Thereafter, GLUT4 translocation was determined. The quantitated values represent the mean  $\pm$  SE of 3–4 experiments. <sup>\*</sup> $P < 0.05$ , <sup>\*\*</sup> $P < 0.01$ , <sup>#</sup> $P < 0.001$ , <sup>##</sup> $P < 0.0001$  compared with basal or as indicated in figure. □, basal; ■,  $\alpha$ -lipoic acid; ▨, insulin.



**FIG. 6.** Activation of PI 3-kinase by insulin and  $\alpha$ -lipoic acid. L6 muscle cells were treated with 100 nmol/l insulin (5 min) or with 2.5 mmol/l  $\alpha$ -lipoic acid (5 or 10 min) in the absence or presence of 100 nmol/l wortmannin. Cells stimulated in the presence of wortmannin were also pretreated with the drug for 30 min. PI 3-kinase activity associated with IRS-1 immunoprecipitates was then determined using an in vitro kinase assay. Results are expressed in relative units and represent the mean  $\pm$  SE (or SD for  $n = 2$ ) of two to five independent experiments. Kinase activity in the absence of any treatment was assigned a value of 1. The reduction in PI 3-kinase activity between basal and lipoic acid-treated cells was statistically significant ( $P < 0.05$  and  $P < 0.01$ , respectively, ANOVA), whereas the difference between basal and wortmannin/lipoic acid-treated cells was not ( $P = 0.54$  and  $P = 0.85$ , respectively, ANOVA).

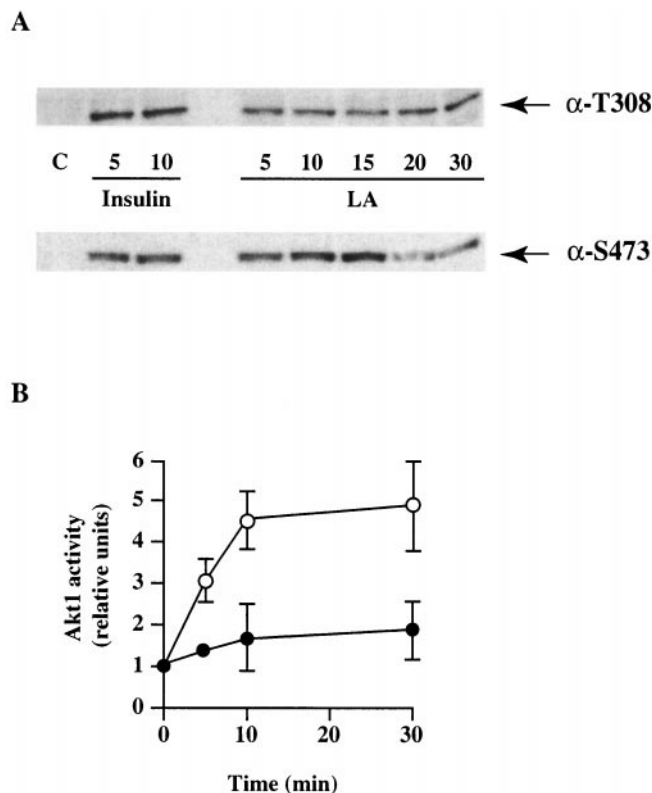
uptake by SB202190/SB203580 was due to a reduction in GLUT4 translocation, we next measured cell surface GLUT4 levels under conditions similar to those described above. Treatment with  $\alpha$ -lipoic acid or insulin alone caused an increase in cell surface GLUT4myc by  $2.9 \pm 0.4$ -fold and  $2.7 \pm 0.2$ -fold, respectively (Fig. 5A). Pretreatment with SB202190 (20  $\mu$ mol/l) had no effect on the translocation of GLUT4myc elicited by  $\alpha$ -lipoic acid ( $\alpha$ -lipoic acid + SB202190:  $3.0 \pm 0.3$ -fold) or insulin (insulin + SB202190:  $3.0 \pm 0.4$ -fold). Similarly, 20  $\mu$ mol/l SB203580 (Fig. 5B) had no effect on GLUT4myc translocation stimulated by either  $\alpha$ -lipoic acid ( $\alpha$ -lipoic acid  $2.8 \pm 0.4$ -fold,  $\alpha$ -lipoic acid + SB203580  $3.0 \pm 0.6$ -fold) or by insulin (insulin  $2.6 \pm 0.2$ -fold, insulin + SB203580  $2.9 \pm 0.7$ -fold).

In contrast with the insensitivity to SB202190 or SB203580, 100 nmol/l wortmannin sharply reduced the GLUT4 translocation caused by  $\alpha$ -lipoic acid ( $\alpha$ -lipoic acid  $2.3 \pm 0.3$ -fold,  $\alpha$ -lipoic acid + wortmannin  $1.3 \pm 0.1$ -fold,  $P = 0.0001$ ). As expected, wortmannin also eliminated the insulin-stimulated GLUT4 translocation (insulin  $2.4 \pm 0.1$ -fold, insulin + wortmannin  $1.1 \pm 0.2$ -fold,  $P < 0.0001$ , ANOVA) (Fig. 5C).

**$\alpha$ -Lipoic acid stimulates PI 3-kinase in a wortmannin-inhibitable manner.** We have previously demonstrated that the stimulation of glucose uptake by  $\alpha$ -lipoic acid is prevented by wortmannin in L6 myotubes (9). However, we have not demonstrated activation of PI 3-kinase by  $\alpha$ -lipoic acid in these cells before. Therefore, we measured the effect of  $\alpha$ -lipoic acid on PI 3-kinase activity. Figure 6 shows that  $\alpha$ -lipoic acid caused a stimulation of IRS-1-associated PI 3-kinase activity by  $20.2 \pm 5.9$ -fold above control within 5 min. The stimulation by insulin at the same time point was  $28.1 \pm 5.0$ -fold above control. After 10 min,  $\alpha$ -lipoic acid-stimulated PI 3-kinase activity was  $30.8 \pm 7.8$ -fold above control. Pretreatment with 100

nmol/l wortmannin inhibited PI 3-kinase by  $\alpha$ -lipoic acid at both time points (5 min wortmannin +  $\alpha$ -lipoic acid  $5.8 \pm 2.1$  fold, 10 min wortmannin +  $\alpha$ -lipoic acid  $2.7 \pm 1.0$  fold). These values were not significantly different from the basal PI 3-kinase activity.

**$\alpha$ -Lipoic acid stimulates Akt1 in a wortmannin-sensitive manner.** The Ser/Thr kinase Akt1 is a downstream effector of PI 3-kinase, and insulin stimulates Akt activity in L6 muscle cells (29,30). Given the potent stimulation of PI 3-kinase by  $\alpha$ -lipoic acid, it was important to examine whether Akt1 was also activated by this compound.  $\alpha$ -Lipoic acid rapidly stimulated T308 and S473 phosphorylation of Akt1 and remained elevated for at least 30 min (Fig. 7A). Figure 7B shows a time course of the activation of Akt1 by  $\alpha$ -lipoic acid, measured by an in vitro kinase assay. Akt1 activity increased by  $3.1 \pm 0.5$ -fold above control after 5 min of stimulation with  $\alpha$ -lipoic acid and was still elevated at 30 min ( $4.9 \pm 1.1$ -fold above control). Maximum activation was observed between 10 and 30 min. Pretreatment with 100 nmol/l wortmannin prevented the activation of Akt1 by  $\alpha$ -lipoic acid at all time points investigated.



**FIG. 7.**  $\alpha$ -Lipoic acid stimulates Akt1 in a wortmannin-sensitive fashion. Total cell lysates were prepared from L6 muscle cells that were treated for the indicated times with or without 100 nmol/l insulin or 2.5 mmol/l  $\alpha$ -lipoic acid. **A:** Lysates (50  $\mu$ g) were resolved by 10% SDS-PAGE and immunoblotted with anti-phospho-T308 or anti-phospho-S473 Akt1-antibody. Representative immunoblot of two independent experiments. **B:** In vitro Akt1 activity assay. Cells stimulated in the presence of wortmannin were also pretreated with the drug for 30 min. Akt1 was the immunoprecipitated, and kinase activity was determined using an in vitro kinase assay. Results are expressed in relative units and represent the mean  $\pm$  SE of three to five independent experiments. Kinase activity in the absence of any treatment was assigned a value of 1. ○,  $\alpha$ -lipoic acid; ●,  $\alpha$ -lipoic acid + wortmannin.

## DISCUSSION

Currently used agents to lower blood glucose in diabetic patients are various sulfonylureas, the biguanide metformin, and an emerging number of thiazolidinediones. Here, we found no effect of metformin on glucose transport, whereas troglitazone and glyburide stimulated glucose uptake moderately when presented to cells in culture for 30 min. Previous studies have examined the potential effect of thiazolidinediones on glucose uptake in diverse systems, with variable results: in a different clone of L6 myotubes, troglitazone did not stimulate glucose uptake acutely, although it increased it after 18 h, presumably through an increase in glucose transporter synthesis (25). In isolated rat cardiomyocytes, two different thiazolidinediones did not stimulate glucose uptake for up to 2 h, but they did potentiate insulin action (31,32). In human muscle cells in culture derived from patients with type 2 diabetes, troglitazone increased glucose uptake after 90 min (33). Finally, in isolated muscle strips, troglitazone increased glucose uptake also after 90 min (34). Regarding glyburide, in an earlier study, we did not see an acute stimulation of glucose uptake in parental L6 myotubes with this drug (24). A higher amount of GLUT4 expression in the cells used in the present study might explain this difference.

The observed stimulation of glucose uptake by the various antidiabetic agents was much less prominent than the effect of  $\alpha$ -lipoic acid. This potent antioxidant was also able to improve insulin-stimulated glucose transport in different animal models of type 1 and 2 diabetes (5,6,8). Treatment of 3T3-L1 adipocytes with this agent resulted in increased glucose uptake and translocation of GLUT1 and GLUT4 (10). Importantly, wortmannin fully inhibited the stimulation of glucose uptake by  $\alpha$ -lipoic acid in 3T3-L1 adipocytes (10) and L6 myotubes (9). Unlike other insulin mimetic agents,  $\alpha$ -lipoic acid utilizes elements of the insulin signaling pathway to elicit translocation of glucose transporters. Therefore, from a therapeutic perspective, it is important to understand the mechanism by which  $\alpha$ -lipoic acid stimulates glucose uptake.

**$\alpha$ -Lipoic acid stimulates GLUT4 translocation in L6 myotubes.** In this study we demonstrate that  $\alpha$ -lipoic acid was able to increase the plasma membrane content of GLUT4 and stimulate glucose uptake in L6 GLUT4myc myotubes to a similar extent as insulin and in a wortmannin-sensitive manner. In our previous study (9), we used subcellular fractionation to detect GLUT4 translocation in L6 myotubes. We have now measured GLUT4 translocation by detecting the presence of a "tagged" GLUT4 protein on the surface of intact cells, e.g., GLUT4myc (20,35). Compared with subcellular fractionation, this method allows the detection of only GLUT4 molecules, which are fully incorporated in the plasma membrane; membrane cross-contamination inherent to the fractionation is not an issue. In addition, reactivity of the myc surface epitope allows the measurement of GLUT4 translocation in intact cells. In L6 muscle cells expressing GLUT4myc, GLUT4 is the major transporter mediating both basal and insulin-stimulated glucose uptake (W. Niu, P. Bilan, T.R., and A.K., unpublished observations). We and others have shown previously that activation of PI 3-kinase (36,37) and its downstream target Akt1 (30,38–40) contribute to GLUT4 translocation to the plasma membrane in muscle and fat

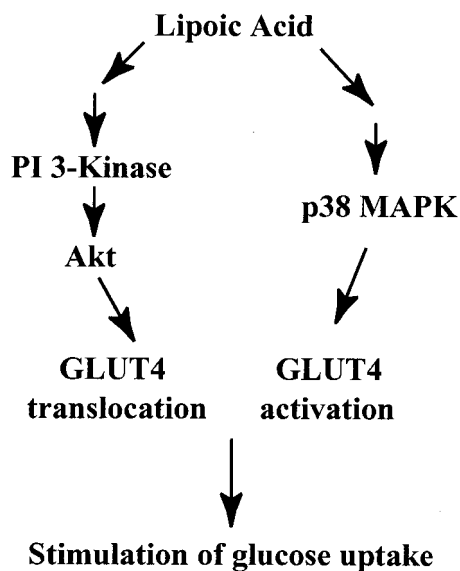
cells in culture. Here we show that  $\alpha$ -lipoic acid stimulated PI 3-kinase and Akt1 activity in L6 myotubes to a similar extent as insulin and in a wortmannin-sensitive manner.

**$\alpha$ -Lipoic acid activates glucose uptake via a p38 MAPK-dependent pathway.** A second signaling pathway elicited by insulin appears to lead to the regulation of GLUT4 activity. This conclusion is based on the fact that pyrimidinyl imidazoles can reduce insulin-stimulated glucose uptake without affecting GLUT4 translocation. Here we further show that 10  $\mu$ mol/l concentrations of the pyrimidinyl imidazoles SB202190 or SB203580 reduced the stimulation of glucose uptake by  $\alpha$ -lipoic acid. It was previously shown that up to 10  $\mu$ mol/l of SB203580 were required to fully inhibit p38 MAPK in intact cells based on cyclic AMP response element binding protein-, ATF2-, and MAPK-activated protein kinase phosphorylation (41–43). This concentration is significantly higher than the concentration needed to inhibit p38 MAPK  $\alpha$  and  $\beta$  in vitro (28).

In contrast to their effect on glucose uptake, SB202190 and SB203580 did not affect the  $\alpha$ -lipoic acid-stimulated exposure of GLUT4 into the plasma membrane. We have previously shown that the insulin-stimulated glucose uptake, but not the translocation of GLUT1 or GLUT4, is reduced by SB203580 in 3T3-L1 adipocytes and L6 myotubes (18). In addition, we have also observed that insulin-stimulated glucose uptake, but not the translocation of GLUT1 or GLUT4, is impaired in 3T3-L1 adipocytes expressing a dominant-negative mutant of p38 MAPK (R.S., T.R., P. Scherer and A.K., unpublished observations). Taken together, these results suggest that GLUT4 translocation is not sufficient to cause maximum stimulation of glucose uptake. The partial inhibition of glucose uptake (47–67%) obtained using the p38 MAPK inhibitors suggest that the translocated transporters are capable of transporting glucose. However, to achieve maximum stimulation of glucose uptake,  $\alpha$ -lipoic acid and insulin increase the intrinsic activity of the transporters. This activation of the cell surface transporters may be mediated by p38 MAPK, perhaps via a pathway independent of PI 3-kinase.

Activation of p38 MAPK by insulin-mimetic agents has not been investigated before. Here we demonstrate that  $\alpha$ -lipoic acid and insulin cause phosphorylation of p38 MAPK. Of the four isoforms of p38 MAPK described so far, only the  $\alpha$  and  $\beta$  isoforms are sensitive to SB203580. Therefore, we determined the effect of  $\alpha$ -lipoic acid and insulin on these two isoforms in L6 GLUT4myc myotubes. Both agents increased the phosphorylation and kinase activity of these two isoforms of p38 MAPK. We have recently demonstrated that insulin and contraction activate p38 MAPK  $\alpha$  and  $\beta$  isoforms in rat skeletal muscle (21). Together, these results suggest a positive correlation between the stimulation of glucose uptake and activation of p38 MAPK. This correlation is strengthened by the results discussed above showing that inhibition of p38 MAPK by various strategies results in reduced glucose uptake.

The pyrimidinyl imidazoles SB202190 and SB203580 used in this study to inhibit p38 MAPK are believed to be specific for p38 MAPK. Neither of these inhibitors, at concentrations that fully inhibit p38 MAPK, has any effect on the activity of closely related kinases, such as extracellular signal-regulated kinase-1 and -2 and the c-Jun NH<sub>2</sub>-terminal kinase (44). We have previously demonstrated



**FIG. 8.** Model of  $\alpha$ -lipoic acid-stimulated glucose uptake. Stimulation of glucose transport by  $\alpha$ -lipoic acid consists of two events: PI 3-kinase-dependent translocation of GLUT4 to the plasma membrane and p38 MAPK-dependent stimulation of their intrinsic activity.

that 10  $\mu\text{mol/l}$  SB203580 does not affect early insulin-dependent signals, such as IRS-1 phosphorylation and activation of PI 3-kinase, Akt1, Akt2, and Akt3 in 3T3-L1 adipocytes (18). Moreover, lack of effect of SB202190 and SB203580 on  $\alpha$ -lipoic acid- and insulin-stimulated GLUT4myc translocation supports the notion that the early signals required to induce GLUT4 translocation were not affected by these drugs.

In conclusion, our data suggest that  $\alpha$ -lipoic acid stimulates glucose uptake by translocating and regulating the intrinsic activity of GLUT4 (Fig. 8). The latter effect may be mediated by stimulating the p38 MAPK or a closely related SB202190- and SB203580-sensitive target. Collectively, these results further support a role of  $\alpha$ -lipoic acid as an antidiabetic agent in the treatment of diabetes.

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This article is dedicated to the memory of Toolsie Ramlal.

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