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Thrombin-induced glucose transport *via* Src–p38 MAPK pathway in vascular smooth muscle cells

*,[1]Yasunari Kanda & [1]Yasuhiro Watanabe

[1]Department of Pharmacology, National Defense Medical College, 3-2 Namiki, Tokorozawa, Saitama 359-8513, Japan

1 Thrombin is a mitogen for vascular smooth muscle cells (VSMC) and has been implicated in the development in atherosclerosis. However, little is known about the role of thrombin in glucose transport in VSMC.

2 In this study, we examined the effect of thrombin on glucose uptake in rat A10 VSMC. We found that thrombin induced glucose uptake in a dose-dependent manner while hirudin, a potent thrombin inhibitor, prevented glucose uptake in the cells. PP2, a selective inhibitor of Src, prevented the thrombin-induced glucose uptake, but did not affect insulin-induced uptake.

3 We also examined whether mitogen-activated protein kinase (MAPK) inhibitors influenced thrombin-induced glucose uptake. The p38 MAPK inhibitor (SB203580) inhibited thrombin-induced glucose uptake, but the MEK inhibitor (PD98059) did not. In contrast to thrombin, SB203580 did not affect insulin-induced glucose uptake. Furthermore, thrombin failed to translocate the insulin-sensitive glucose transporter GLUT4.

4 These findings suggest that thrombin stimulates glucose transport *via* Src and subsequent p38 MAPK activation in VSMC.
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Keywords: Thrombin; insulin; vascular smooth muscle cells; Src; p38 MAPK; glucose uptake

Abbreviations: DMEM, Dulbecco's modified Eagle's medium; EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; GPCR, G protein-coupled receptor; LPA, lysophosphatidic acid; MAPK, mitogen-activated protein kinase; PM, plasma membrane; PMT, pasteurella multocida toxin; PTX, pertussis toxin; VSMC, vascular smooth muscle cells

Introduction

Vascular smooth muscle cells (VSMC) play a key role in the pathogenesis of atherosclerosis and restenosis after percutaneous transluminal coronary angioplasty (Ross, 1999). Thrombin is a multifunctional serine protease and stimulation of cells with thrombin leads to protein phosphorylation, gene expression, contractility and proliferation in a variety of cells, including VSMC (Patterson *et al*., 2001). These effects are mediated by the thrombin receptor, which has been shown to be a member of G protein-coupled receptors (GPCRs). Several lines of evidence in animal models suggest a possible role of thrombin in response to injuries that result in vascular lesion formation. For example, administration of hirudin, a potent thrombin inhibitor, reduces neointimal formation after balloon injury in rabbits (Sarembock *et al*., 1991).

Glucose is a metabolic energy for cell proliferation and a family of glucose transporters has already been identified (Joost & Thorens, 2001). Among them, GLUT1 and GLUT4 have been shown to be expressed in VSMC. GLUT1, which is widely expressed in various cells of the body, is thought to be the predominant transporter in VSMC (Klip *et al*., 1994; Quinn & McCumbee, 1998). GLUT4 is an insulin-responsive isoform and is expressed in muscle and fat cells. Insulin stimulation results in glucose uptake through the translocation of GLUT4 from an intracellular membrane compartment to the plasma membrane (PM; Czech & Corvera, 1999; Pessin *et al*., 1999). However, it is unclear whether thrombin affects glucose transport in VSMC.

In this study, we examined the role of thrombin in glucose transport in cultured rat A10 VSMC, which adopt a phenotype similar to that of the VSMC observed in the atherosclerosis plaque. We report here that thrombin stimulates glucose uptake *via* a Src–p38 MAPK-dependent mechanism.

Methods

Cell culture

A10 cells (rat thoracic aortic smooth muscle cells) were provided by the American Type Cell Collection (Rockville, MD, U.S.A.; CRL 1476). The cells were cultured at 37°C in 100 mm dishes in a humidified atmosphere of 5% CO_2/95% air. The growth medium comprised Dulbecco's modified Eagle's medium (DMEM; Nissui Pharmaceutical Co., Ltd, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS; JRH Biosciences, Lenexa, KS, U.S.A.), penicillin (100 U ml^{-1}; Gibco BRL, Gaithersburg, MD, U.S.A.), and streptomycin (100 μg ml^{-1}; Gibco BRL). The medium was changed twice a week and cell passages 18–24 were used for all experiments.

*Author for correspondence; E-mail: kanda@ndmc.ac.jp

Plasmids

Plasmids encoding constitutively active $G\alpha_q$ (G_qQ209L) were kindly donated by J.H. Exton (Vanderbilt University School of Medicine, Nashville, TN, U.S.A.).

Adenovirus infection

A recombinant adenovirus encoding green fluorescent protein (GFP) and GFP-phosducin was kindly donated by Dr H. Kurose (University of Kyusyu, Japan). Cells in 24-well plates were infected with the adenovirus at a multiplicity infection of 100 in serum-free DMEM. After incubation for 1 h, cells were incubated in DMEM containing 10% FBS. All experiments were performed 48 h after infection.

Transient transfection of expression plasmids

The plasmids were transfected into A10 cells (70–80% confluency) using LipofectAMINE™ (Gibco BRL) according to manufacturer's recommendation. The total amount of DNA was adjusted to 2 μg per 60-mm dish.

Cell lysis, immunoprecipitation, and immunoblotting

The cells were serum-starved for 24 h in serum-free DMEM. The cells were stimulated with agonists for the indicated times at 37°C and lysed in a buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 10 mM $Na_4P_2O_7$, 20 mM NaF, 2 mM EDTA, 1% Triton X-100, 1 mM PMSF, 1 mM Na_3VO_4, 10 μg ml^{-1} aprotinin, and 10 μg ml^{-1} leupeptin). Following incubation on ice for 30 min, the lysed cells were centrifuged at 15,000 $\times$ *g* for 20 min at 4°C to precipitate debris. The supernatant was collected and assayed for protein concentration using a BCA Protein Assay Reagent Kit (Pierce, Rockford, IL, U.S.A.). For immunoprecipitation, the supernatant was precleared with protein G sepharose beads (Amersham Pharmacia Biotech, Buckinghamshire, U.K.) and incubated with the appropriate antibody conjugated to sepharose beads overnight at 4°C. The samples were analyzed on 12% SDS–PAGE and transferred electrophoretically to PVDF membranes (15 V, 90 min; Millipore, Bedford, MA, U.S.A.). After blocking in 5% skim milk in PBS-T (0.2% Tween 20) for 1 h at room temperature, membranes were reacted with specific antibodies overnight at 4°C. The blots were then washed and then incubated with HRP-conjugated secondary antibodies (Calbiochem; 1 : 2000 dilution) for 1 h at room temperature. After washing, the signal was detected by enhanced chemiluminescence (ECL detection kit; Amersham Pharmacia Biotech).

p38 MAPK activity assay

p38 MAPK activity in immunoprecipitates was measured using the p38 MAPK assay kit (Cell Signaling Technology, Beverly, MA, U.S.A.), as reported previously (Kanda *et al*., 2001a). In brief, p38 MAPK was immunoprecipitated from cell lysates using 2 μg of anti-p38 MAPK antibody conjugated to sepharose beads overnight at 4°C. The immunoprecipitates were washed twice with a lysis buffer and twice with a kinase buffer (20 mM Tris, pH 7.4, 20 mM $MgCl_2$, 20 mM NaCl, 0.1 mM Na_3VO_4, 2 mM DTT). The beads were then suspended in 50 μl of the kinase buffer containing 2 μg GST-ATF-2, 20 μM ATP at 30°C for 30 min. Reactions were stopped by adding 5 $\times$ Laemmli sample buffer and heating for 5 min. Phosphorylation of ATF-2 was analyzed by immunoblotting using anti-phospho-specific ATF-2 antibody (1 : 2000 dilution).

Glucose uptake assay

Glucose uptake was determined using 2-[^{3}H]deoxy-D-glucose (2-DG; Amersham Pharmacia Biotech), a nonmetabolizable analogue of glucose, as reported by Low *et al*. (1992). In brief, A10 cells grown in 24-well plates were incubated in serum-free DMEM for 24 h before the assay was performed. After exposure to agonists for 30 min, the cells were washed three times with prewarmed phosphate-buffered saline (PBS) and incubated with 2-DG (10 μM, 1 μCi ml^{-1}) for 20 min. Under these conditions, glucose uptake was linear for at least 20 min (data not shown). The uptake was terminated by washing the cells three times with ice-cold PBS. The cells were solubilized in 0.1 N NaOH/0.1% SDS for 1 h. Radioactivity was measured by liquid scintillation spectroscopy (Aloka, Tokyo, Japan). All experiments were performed in triplicate. Nonspecific uptake was measured in the presence of 100 mM of glucose or 100 μM cytochalasin B.

Cell fractionation

PM fractions were prepared using the differential centrifugation method as previously reported (Rudich *et al*., 1998). In brief, A10 cells were incubated with the appropriate agonists. After washing with PBS, the cells were lysed with a lysis buffer (20 mM HEPES, pH 7.4, 1 mM EDTA, 250 mM sucrose, 2 mM Na_3VO_4, 10 mM NaF, 1 mM $Na_4P_2O_7$, 1 mM PMSF, 10 μg ml^{-1} aprotinin, and 10 μg ml^{-1} leupeptin). The lysates were centrifuged at 2000 $\times$ *g* for 20 min to remove mitochondria and nuclei. The resultant supernatant was then centrifuged at 18,000 $\times$ *g* for 20 min to pellet the crude PM fractions. The crude fractions were washed with a lysis buffer to exclude any contamination by the supernatant.

Statistics

Values are expressed as the arithmetic means $\pm$ s.d. Statistical analysis of the data was performed by the use of one-way analysis of variance (ANOVA), followed by Scheffe test when *F*-ratios were significant ($P<0.05$).

Materials

Thrombin, lysophosphatidic acid (LPA), PD98059, and CRM197 were from Sigma-Aldrich (St Louis, MO, U.S.A.). Pertussis toxin (PTX) was from Funakoshi (Tokyo, Japan). Anti-GLUT-1 and anti-GLUT-4 were from Chemicon International (Temecula, CA, U.S.A.). Anti-p38 MAPK polyclonal antibody, anti-phospho-tyrosine monoclonal antibody (PY20), and anti-epidermal growth factor receptor (EGFR) antibody were from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Insulin was from Nacalai Tesque (Kyoto, Japan). Anti-phospho-specific Src [pY418] antibody was from Biosource International (Camarillo, CA, U.S.A.). Hirudin, Pasteurella multocida toxin (PMT), AG1478, PP2, SB203580, and SB202474 were from Calbiochem (La Jolla,

CA, U.S.A.). U0126 was from Promega (Madison, WI, U.S.A.). All other reagents were of analytical grades and obtained from commercial sources.

Results

Thrombin stimulates glucose uptake in A10 cells

To examine whether thrombin stimulates glucose uptake in VSMC, A10 cells were exposed to various concentrations of thrombin for 30 min. As shown in Figure 1a, thrombin stimulates glucose uptake in a dose-dependent manner, with the maximum response observed at 0.1 U ml^{-1}. To confirm that the effect was mediated by a receptor-dependent mechanism, we treated the cells with hirudin, which inhibits the binding of thrombin to its receptor (Wallis, 1996). Hirudin inhibited thrombin-stimulated glucose uptake to the basal level (Figure 1b). These data suggest that thrombin stimulates glucose uptake through its receptor in VSMC.

Glucose uptake by thrombin is mediated via *α-subunit of PTX-insensitive G proteins*

We next examined the signaling pathways from the thrombin receptor to glucose uptake in A10 cells. Previous studies have shown that the thrombin receptor is coupled to the G_i, G_q, G_{12} classes of heterotrimeric G proteins (LaMorte *et al*., 1993; Offermanns *et al*., 1994). To examine the involvement of G_i in glucose uptake, we pretreated cells with PTX, which ADP-ribosylates G_i and inhibits G_i signaling. PTX did not affect the thrombin-stimulated glucose uptake, but inhibited LPA-induced glucose uptake (Figure 2). These results suggest that PTX-sensitive G proteins do not mediate thrombin-induced glucose uptake in VSMC.

After a ligand binds its GPCR and stimulates heterotrimeric G proteins, Gα and Gβγ are dissociated and both of them can mediate signals. To determine whether Gβγ was involved in thrombin-stimulated glucose uptake, we used the adenoviral gene-transfer method (Nishida *et al*., 2000) to deliver phosducin, which is known to bind Gβγ and inhibit its signaling. As shown in Figure 3, the expression of phosducin had no effect on thrombin-stimulated glucose uptake. The effectiveness of phosducin was confirmed by the significant inhibition of H_2O_2-induced ERK phosphorylation. Taken together, these data suggest that thrombin stimulates glucose uptake *via* the α-subunit of PTX-insensitive G protein(s).

Inhibition of Src family kinase(s) blocks thrombin-induced glucose uptake in A10 cells

The activation of GPCRs reportedly requires tyrosine kinase activity (Wan *et al*., 1996). However, GPCRs, including the thrombin receptor, do not possess intrinsic tyrosine kinase activity. Since thrombin can activate both nonreceptor tyrosine kinase (Src) and receptor-type tyrosine kinase (EGFR) (Ishida *et al*., 1999; Kanda *et al*., 2001a), we examined whether these tyrosine kinases regulate thrombin-induced glucose uptake in A10 cells. As shown in Figure 4a, PP2, a highly selective inhibitor for Src family kinase(s) (Hanke *et al*., 1996), blocked thrombin-induced glucose uptake, but had no effect on the insulin-induced glucose uptake. The effectiveness of PP2 was confirmed by immunoblot with anti-phospho-specific Src [pY418] antibody. The tyrosine 418 is known to be essential for Src activation (Brown & Cooper, 1996). PP2 inhibited thrombin-induced Src phosphorylation (Figure 4b).

In contrast, AG1478, a selective inhibitor of EGFR kinase, had no effect on the glucose uptake (Figure 5a). CRM197, which inhibits thrombin-induced EGFR transactivation (Kalmes *et al*., 2000), also failed to affect the glucose uptake

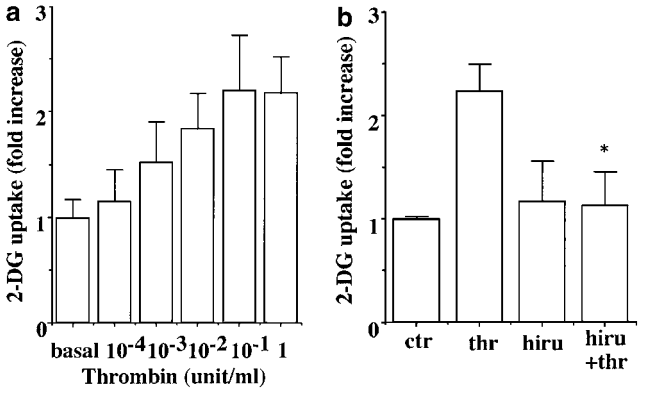

Figure 1 Effect of thrombin on 2-DG uptake in A10 cells. A10 cells in 24-well plates were serum-starved for 24 h. The cells were stimulated for 30 min with various concentrations of thrombin (a) or with 1 U ml^{-1} of thrombin in the presence or absence of hirudin (b), and were evaluated by assay for 2-DG uptake, as described under Methods. Each value represents the mean ± s.d. of three independent experiments in triplicate. *$P<0.05$ as compared with the respective control.

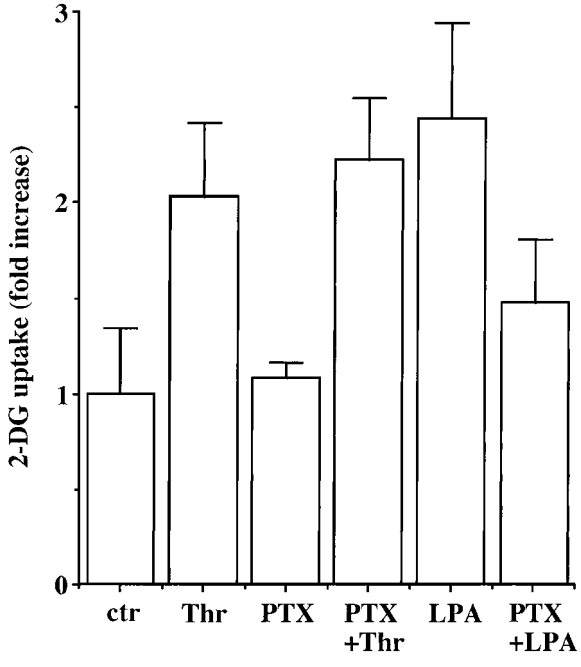

Figure 2 Effect of PTX on thrombin-induced 2-DG uptake in A10 cells. A10 cells were incubated with or without PTX (50 ng ml^{-1}) for 24 h in serum-free DMEM. The cells were stimulated with thrombin (1 U ml^{-1}) or LPA (10 μM) for 30 min and were evaluated by assay for the 2-DG uptake, as described under Methods. Each value represents the mean ± s.d. of three independent experiments in triplicate.

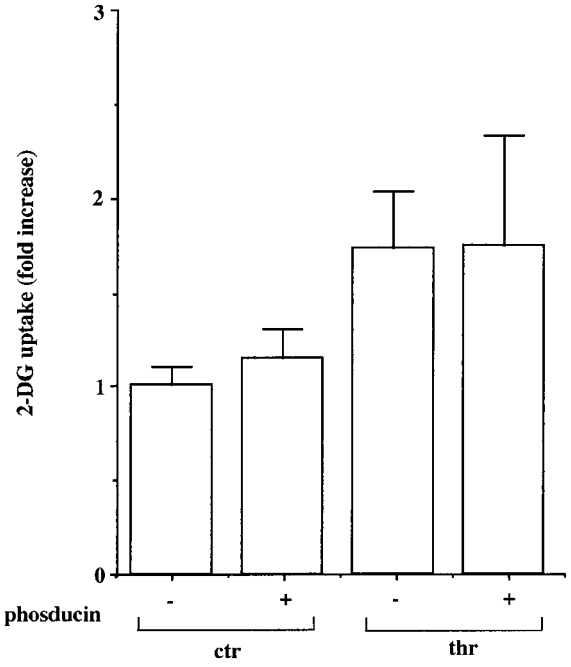

Figure 3 Effects of phosducin on thrombin-induced 2-DG uptake in A10 cells. A10 cells were infected with phosducin and incubated for 24 h. After the cells were serum-starved for further 24 h, the cells were stimulated with thrombin (1 U ml^{-1}) for 30 min and were evaluated by assay for the 2-DG uptake as described under Methods. Each value represents the mean ± s.d. of three independent experiments in triplicate.

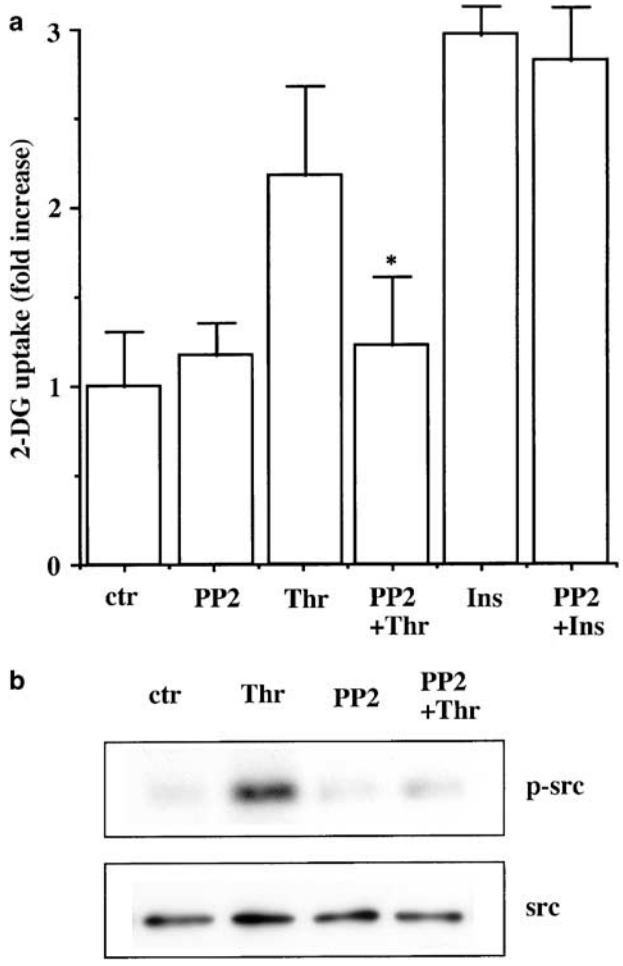

Figure 4 Effects of PP2 on thrombin-induced 2-DG uptake and Src phosphorylation in A10 cells. Serum-starved A10 cells were incubated with or without PP2 (1 μM) for 30 min. (a) The cells were stimulated with thrombin (1 U ml^{-1}) or insulin (1 μM) for 30 min and were evaluated by assay for the 2-DG uptake, as described under Methods. Each value represents the mean ± s.d. of three independent experiments in triplicate. *$P<0.05$ as compared with the respective control. (b) The cells were stimulated with thrombin (1 U ml^{-1}) for 5 min and lysed. Src phosphorylation was analyzed by immunoblotting with anti-phospho-specific Src antibody.

in A10 cells. Both AG1478 and CRM197 inhibited thrombin-induced EGFR phosphorylation (Figure 5b). These data suggest that thrombin-induced glucose uptake is mediated through Gα–Src family kinase(s), not the EGFR transactivation pathway, in VSMC.

PMT-induced glucose uptake in A10 cells

To evaluate the role of α-subunit of G_q in glucose uptake, we tested the effect of PMT, which is known to be internalized by endocytosis and act intracellularly to activate free monomeric $G\alpha_q$ (Murphy & Rozengurt, 1992; Wilson *et al*., 1997). As shown in Figure 6a, exposure to PMT stimulated glucose uptake in a dose-dependent manner. Moreover, PP2 inhibited PMT-induced glucose uptake (Figure 6b), suggesting that PMT stimulates glucose uptake *via* Src family kinase(s). To further confirm that $G\alpha_q$ is involved in Src activation, we examine the effect of constitutively active mutant of $G\alpha_q$ on Src phosphorylation. Transient expression of constitutively active $G\alpha_q$ caused Src phosphorylation in A10 cells (Figure 6c). Collectively, these data suggest that $G\alpha_q$–Src family kinase(s) mediate thrombin-induced glucose uptake in VSMC.

Effects of MAPK inhibitors on thrombin-induced glucose uptake in A10 cells

We next investigate the downstream signaling from Src activation to glucose uptake. Previously, we reported that a tyrosine kinase plays a role in thrombin-induced p38 MAPK activation in A10 cells (Kanda *et al*., 2001b). Therefore, we examine the role of MAPKs in glucose uptake. SB203580, a selective inhibitor of p38 MAPK, attenuate the thrombin-induced glucose uptake in A10 cells, while SB202474, an inactive structural analog of SB203580, had no effect on the glucose uptake (Figure 7a). SB203580 did not inhibit the insulin-induced glucose uptake. In contrast, PD98059, a selective inhibitor of MEK, which is an upstream kinase of ERK, failed to affect the glucose uptake (Figure 7b). The use of U0126, another MEK inhibitor, produced similar results. The effectiveness of PD98059 was confirmed by its ability to inhibit thrombin-induced ERK phosphorylation (Figure 7c). In addition, thrombin-induced p38 MAPK activation was blocked by PP2, suggesting that Src is an upstream kinase of p38 MAPK (Figure 7d). Taken together, these results suggest that p38 MAPK, not ERK, mediates thrombin-induced glucose uptake in VSMC.

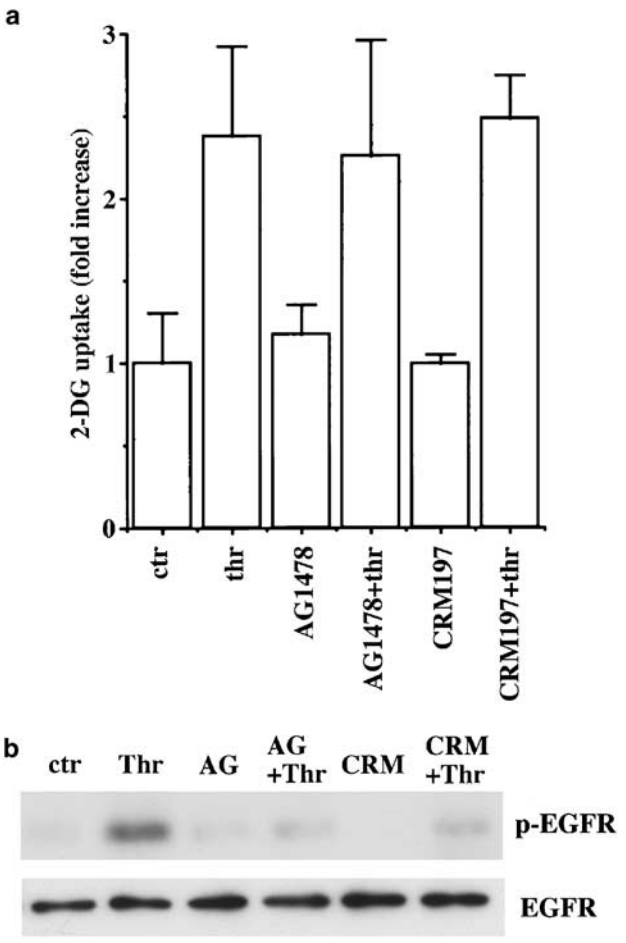

Figure 5 Effects of AG1478 and CRM197 on thrombin-induced 2-DG uptake and EGFR phosphorylation in A10 cells. Serum-starved A10 cells were incubated with or without PP2 (1 μM) for 30 min. (a) The cells were stimulated with thrombin (1 U ml^{-1}) or insulin (1 μM) for 30 min and were evaluated by assay for the 2-DG uptake, as described under Methods. Each value represents the mean ± s.d. of three independent experiments in triplicate. (b) The cells were stimulated with thrombin (1 U ml^{-1}) for 5 min and lysed. After immunoprecipitation with anti-EGFR antibody, the samples were analyzed by immunoblotting with anti-phospho-tyrosine antibody or anti-EGFR antibody.

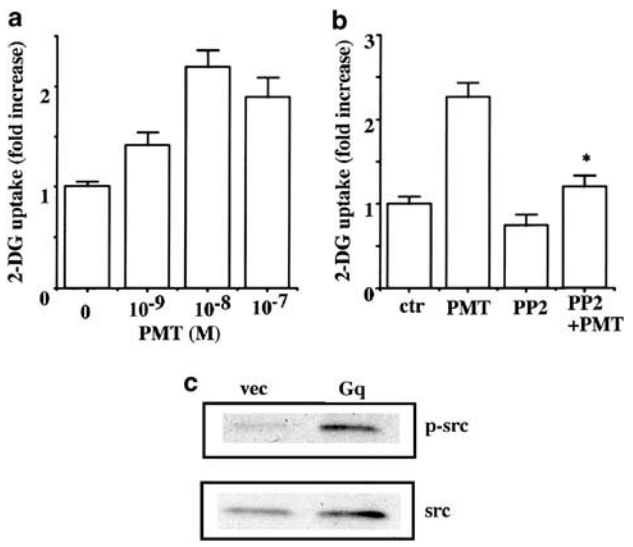

Figure 6 Role of Gα_q in 2-DG uptake in A10 cells. (a) A10 cells were exposed to various concentrations of PMT for 16 h in serum-free DMEM. (b) A10 cells were exposed to PMT for 16 h and then incubated with or without PP2 (1 μM) for 30 min. The cells were evaluated by assay for the 2-DG uptake, as described under Methods. Each value represents the mean ± s.d. of three independent experiments in triplicate. $*P<0.05$ as compared with the respective control. (c) A10 cells were transfected with constitutively active mutant of Gα_q. After 48 h, the cells were lysed and immunoblotted with anti-phospho-specific Src antibody.

Effect of thrombin on glucose transporter translocation

We also examined the influence of GLUT on glucose uptake in A10 cells. Insulin stimulates glucose transport by translocation of GLUT4 to PM in muscle and fat cells. VSMC have been reported to express GLUT1 and GLUT4. To examine whether thrombin translocates GLUT in A10 cells, PMs were prepared by differential centrifugation. As shown in Figure 8a, thrombin did not translocate GLUT1 or GLUT4 in A10 cells. On the other hand, insulin was observed to translocate GLUT4 in A10 cells, suggesting that this cell line can undergo GLUT translocation. The levels of GLUT did not change before and after thrombin stimulation. Pretreatment with cycloheximide, an inhibitor of protein synthesis, failed to affect thrombin-induced glucose uptake (Figure 8b). These data suggest that thrombin does not stimulate glucose uptake due to the cellular distribution and protein synthesis of GLUT.

Additive effect of thrombin and insulin on glucose uptake

To further examine whether thrombin and insulin utilize different signaling pathways to transport glucose, we tested the effect of combination of thrombin and insulin on glucose uptake. When the cells were treated with thrombin plus insulin, the glucose uptake was additive (Figure 9). These data suggest that glucose uptake is differentially regulated by thrombin and insulin.

Discussion

In the present study, we demonstrate a potential role of thrombin in glucose uptake in VSMC. We also show that Src and subsequent p38 MAPK activation plays a role in thrombin-mediated glucose uptake in VSMC. According to the data shown in this study, a proposed model regarding thrombin-mediated glucose transport is shown in Figure 10.

Thrombin exerts many responses by activating heterotrimeric G proteins, G_i, G_q, and G_{12}, which transduce signals through both α and $\beta\gamma$ subunits. Since sequestration of G$\beta\gamma$ did not affect the glucose uptake (Figure 3), we investigated the involvement of Gα in thrombin-induced glucose uptake. We showed that the PTX insensitive G protein, G_q, and G_{12} mediated thrombin-induced glucose uptake (Figure 4). In addition, we found that exposure to PMT, which potently mimics the Gα_q signaling, stimulated glucose uptake in A10 cells.

In the light of these observations, we hypothesize that a linkage exists between Gα_q and glucose uptake in VSMC. Such a connection could explain the relationship between the thrombin effect and the PMT–Gα_q pathway. In 3T3-L1 adipocytes, Gα_q has been shown to be required for glucose

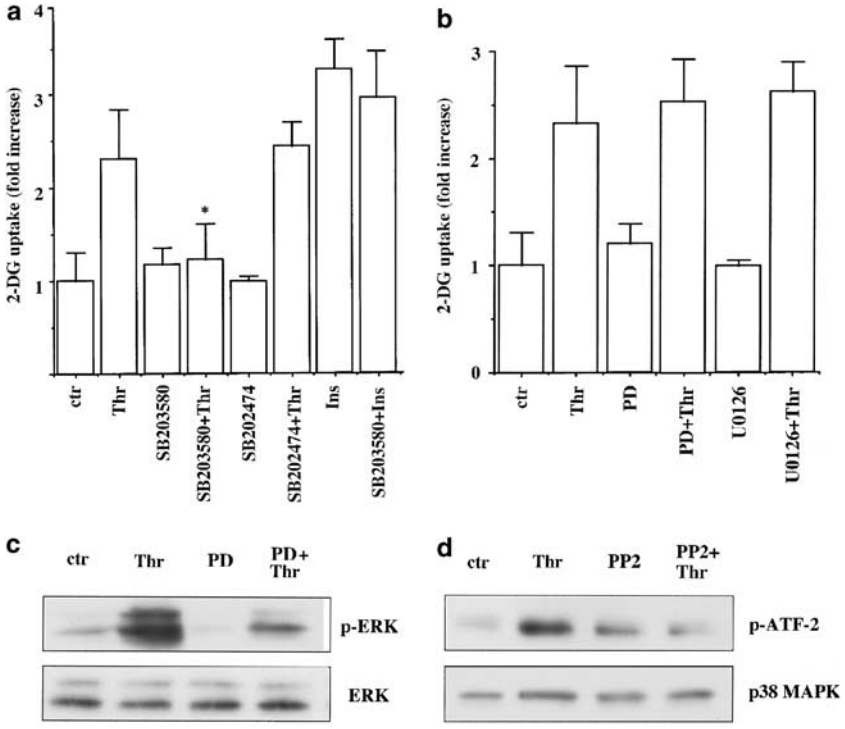

Figure 7 Role of p38 MAPK on thrombin-induced 2-DG uptake in A10 cells. Serum-starved A10 cells were stimulated with thrombin (1 U ml^{-1}) or insulin (1 μM) in the presence or absence of (a) SB203580 (10 μM) and SB202474 (10 μM), (b) PD98059 (10 μM) and U0126 (1 μM). The cells were evaluated by assay for the 2-DG uptake, as described under Methods. Each value represents the mean $\pm$ s.d. of three independent experiments in triplicate. *$P<0.05$ as compared with the respective control. (c) Serum-starved A10 cells were incubated with or without PD98059 (10 μM) for 30 min and then stimulated with thrombin (1 U ml^{-1}) for 5 min. ERK phosphorylation was analyzed by immunoblotting with anti-phospho-specific ERK antibody. (d) Serum-starved A10 cells were incubated with or without PP2 (1 μM) for 30 min and then stimulated with thrombin (1 U ml^{-1}) for 10 min. p38 MAPK activity was assayed, as described under Methods.

uptake induced by endothelin, a GPCR agonist (Imamura *et al*., 1999). Therefore, $G\alpha_q$ might be a regulator of glucose uptake in various cells. Alternatively, since PMT has an ability to activate the rho–rho kinase pathway (Essler *et al*., 1998), $G\alpha_{12}$ could be another target for PMT. Future studies will be needed to explore more carefully the potential involvement of $G\alpha_{12}$ in glucose uptake.

Many lines of evidence indicate that GPCRs can initiate crosstalk with tyrosine kinases. Src can be activated by various GPCR agonists, such as angiotensin II and thrombin (Ishida *et al*., 1999). Furthermore, the expression of a constitutively active mutant of $G\alpha_q$ has induced Src phosphorylation in A10 cells, suggesting that Src acts as a downstream component of $G\alpha_q$. Therefore, we focus on the potential involvement of Src in GPCR-mediated glucose uptake by evaluating the effects of PP2, which has been used to evaluate the role of Src family kinase(s). We found that PP2 inhibited thrombin-induced glucose uptake (Figure 4). However, PP2 did not inhibit insulin-induced glucose uptake. These data suggest that thrombin and insulin utilize different signaling pathways to glucose uptake. This finding is supported by the recent finding that endothelin increases glucose uptake and PP2 blocked the response in 3T3-L1 adipocytes (Hall *et al*., 2001). Thus, Src appears to play a key role in glucose uptake induced by GPCR agonists. Although it is unlikely that PP2 inhibits other kinases, we cannot exclude the possibility that PP2 inhibit other targets. In contrast, EGFR transactivation, another tyrosine kinase pathway, did not appear to be involved in the glucose uptake (Figure 4). Although Src has been shown to link GPCR and EGFR (Eguchi *et al*., 1998), glucose uptake by thrombin is thought to be mediated by a pathway other than EGFR transactivation pathway.

Our further examination of the signal transduction pathways from the Src to glucose uptake revealed that thrombin-induced glucose uptake is inhibited by SB203580, a selective p38 MAPK inhibitor. The role of p38 MAPK in glucose uptake is controversial. Previous studies showed that insulin-induced glucose uptake is suppressed by SB203580 in 3T3-L1 adipocytes (Sweeney *et al*., 1999). However, another study showed that kinase-inactive p38 MAPK did not inhibit insulin-induced glucose uptake in 3T3-L1 adipocytes (Fujishiro *et al*., 2001). Here we found that SB203580 did not inhibit insulin-induced glucose uptake and an inactive structural analog of SB203580, SB202474, did not inhibit the uptake, suggesting that the inhibition by SB203580 might not be due to its nonspecific effects. In contrast to p38 MAPK, ERK did not seem to be involved in thrombin-induced glucose uptake. Since ERK is thought to be mediated from Src (Della Rocca *et al*., 1999), this difference between p38 MAPK and ERK involvement could be due to the downstream components. Akt is another candidate to link between Src and glucose uptake. Thrombin has been shown to induce Akt activation in platelet (Kroner *et al*., 1997) and Src-mediated Akt activation in Chinese hamster ovary embryo fibrablasts (Goel *et al*., 2002). In spite of the difference of cell type, Akt may play a role in the regulation of glucose uptake in VSMC.

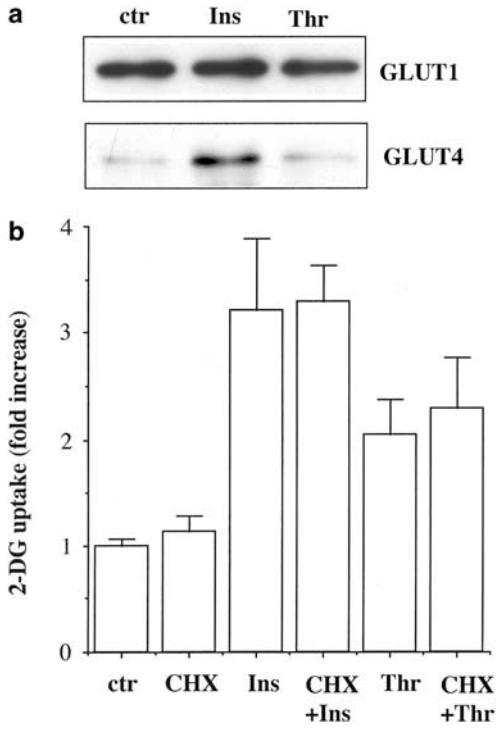

Figure 8 Thrombin did not translocate GLUT4 in A10 cells. (a) Serum-starved A10 cells were stimulated with thrombin (1 U ml^{-1}) or insulin (1 μM) for 20 min. PM fractions were prepared by centrifugation and immunoblotted with anti-GLUT1 or anti-GLUT4 antibody, as described under Methods. (b) Serum-starved A10 cells were incubated with or without cycloheximide (CHX; 10 μM) for 30 min. The cells were stimulated with thrombin (1 U ml^{-1}) or insulin (1 μM) for 30 min and were evaluated by assay for the 2-DG uptake, as described under Methods. Each value represents the mean $\pm$ s.d. of three independent experiments performed in triplicate.

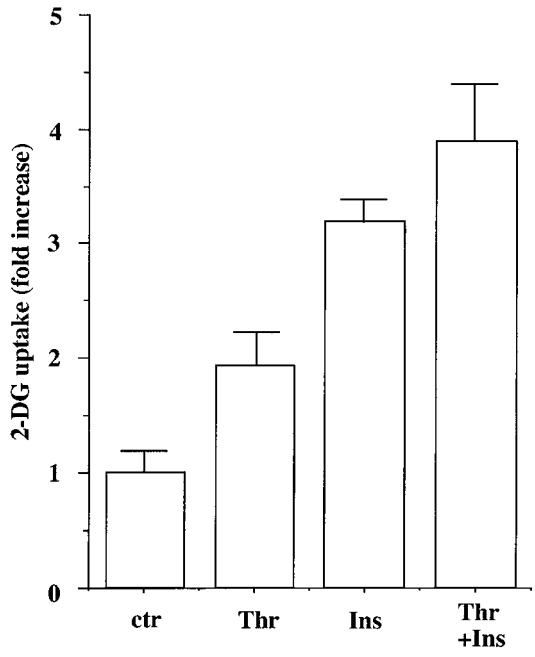

Figure 9 Stimulation of 2-DG uptake by thrombin and insulin in A10 cells. Serum-starved A10 cells were stimulated with thrombin (1 U ml^{-1}), insulin (1 μM), and the combination of thrombin plus insulin for 30 min and were evaluated by assay for the 2-DG uptake, as described under Methods. Each value represents the mean $\pm$ s.d. of three independent experiments in triplicate.

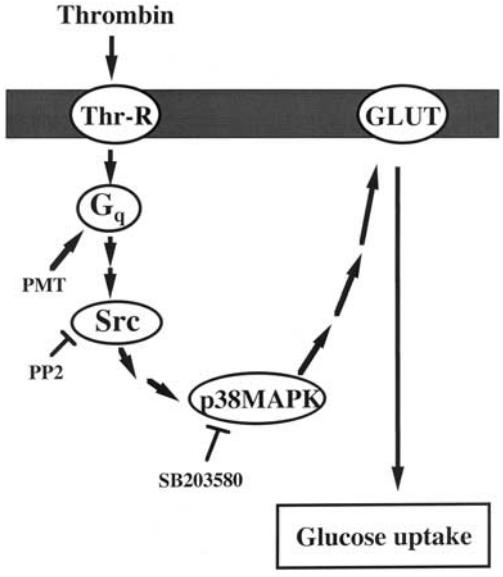

Figure 10 Schematic model summarizing our findings. Src and subsequent p38 MAPK activation plays a role in thrombin-mediated glucose uptake in VSMC.

To further assess the downstream of MAPKs, we analyze the glucose transporter in thrombin-mediated glucose uptake. We observed GLUT4 translocation by insulin in the A10 cells. In contrast to insulin, thrombin failed to translocate GLUT4. Our findings indicate that thrombin-induced glucose uptake is not mediated by GLUT4. GLUT1, which is expressed in VSMC, has been shown to be regulated by transcription (Quinn & McCumbee, 1998). We found that the use of a protein synthesis inhibitor had no effect on thrombin-induced glucose uptake. Moreover, the stimulation time by thrombin is too short to synthesize proteins. This finding suggests that intrinsic activation of GLUT might facilitate glucose uptake by thrombin. It is possible that Src and p38 MAPK regulate this intrinsic activity by a phosphorylation-dependent mechanism. The additive effects of thrombin and insulin on glucose uptake support the notion that thrombin and insulin elicit different signaling pathways to transport glucose. We cannot rule out the possibility that the other isoform of GLUT mediates the thrombin-induced glucose uptake in VSMC. RNA interference technology has recently been shown to be a powerful tool to analyze glucose uptake (Jiang *et al*., 2003; Katome *et al*., 2003). Experiments using RNA interference are now under way to determine the subtype of GLUT.

In summary, the results of this study suggest that thrombin stimulates glucose uptake in VSMC. In addition, Src activation and subsequent p38 MAPK activation play a role in thrombin-mediated glucose uptake. These findings may contribute to an improved understanding of the pathogenesis of atherosclerosis.

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