Signal Transduction: The Glutamate Agonist Homocysteine Sulfinic Acid Stimulates Glucose Uptake through the Calcium-dependent AMPK-p38 MAPK-Protein Kinase C ζ Pathway in Skeletal Muscle Cells

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J. Biol. Chem. 2011, 286:7567-7576.
doi: 10.1074/jbc.M110.149328 originally published online December 30, 2010

Access the most updated version of this article at doi: 10.1074/jbc.M110.149328

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The Glutamate Agonist Homocysteine Sulfinic Acid Stimulates Glucose Uptake through the Calcium-dependent AMPK-p38 MAPK-Protein Kinase C ζ Pathway in Skeletal Muscle Cells*

Received for publication, June 4, 2010, and in revised form, December 22, 2010. Published, JBC Papers in Press, December 30, 2010, DOI 10.1074/jbc.M110.149328

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Homocysteine sulfinic acid (HCSA) is a homologue of the amino acid cysteine and a selective metabotropic glutamate receptor (mGluR) agonist. However, the metabolic role of HCSA is poorly understood. In this study, we showed that HCSA and glutamate stimulated glucose uptake in C2C12 mouse myoblast cells and increased AMP-activated protein kinase (AMPK) phosphorylation. RT-PCR and Western blot analysis revealed that C2C12 expresses mGluR5. HCSA transiently increased the phosphorylation of AMPK, MAPK, and inhibition of AMPK and p38 MAPK blocked HCSA-mediated phosphorylation of p38 MAPK. Expression of dominant-negative CaMKK blocked HCSA-induced AMPK phosphorylation. Pretreatment of cells with STO-609, a calmodulin-dependent protein kinase inhibitor, blocked HCSA-induced AMPK phosphorylation, and knockdown of CaMKK blocked HCSA-induced AMPK phosphorylation. In addition, HCSA activated p38 mitogen-activated protein kinase (MAPK). Expression of dominant-negative AMPK suppressed HCSA-mediated phosphorylation of p38 MAPK, and inhibition of AMPK and p38 MAPK blocked HCSA-induced glucose uptake. Phosphorylation of protein kinase C ζ (PKCζ) was also increased by HCSA. Pharmacologic inhibition or knockdown of p38 MAPK blocked HCSA-induced PKCζ phosphorylation, and knockdown of PKCζ suppressed the HCSA-induced increase of cell surface GLUT4. The stimulatory effect of HCSA on cell surface GLUT4 was impaired in FITC-conjugated PKCζ siRNA-transfected cells. Together, the above results suggest that HCSA may have a beneficial role in glucose metabolism in skeletal muscle cells via stimulation of AMPK.

Homocysteine, a sulfur-containing amino acid, is a potent and selective metabotropic glutamate receptor agonist (1). A number of epidemiologic studies have shown that moderate hyperhomocysteinemia is a risk factor for atherosclerosis (2, 3), stroke (4), Alzheimer disease (5), and schizophrenia (6). Hyperhomocysteinemia is common in patients with non-insulin-dependent diabetes mellitus (7) and nephritic diabetes (8), suggesting a possible relationship between homocysteine and the pathogenesis. Recent evidence has indicated the presence of glutamate receptors in non-neuronal tissues, including skeletal muscle cells; however, the functional implications involving homocysteine in diabetes are not fully understood.

AMP-activated protein kinase (AMPK) is a phylogenetically conserved intracellular energy sensor that plays a central role in the regulation of glucose and lipid metabolism. AMPK, a heterotrimeric complex composed of a catalytic subunit and two regulatory subunits, is activated when cellular energy is depleted (9). Upon activation by allosteric binding of AMP or phosphorylation at Thr172 of the catalytic subunit, AMPK accelerates ATP-generating catabolic pathways, including glucose and fatty acid oxidation (10–12), while simultaneously reducing ATP-consuming anabolic pathways, including cholesterol, fatty acid, and triacylglycerol synthesis (13).

Some nonessential amino acids and derivatives, such as L-glutamate, L-aspartate, and γ-aminobutyric acid, have long been known to act as neurotransmitters (14). In addition, a group of sulfur-containing amino acids has previously been shown to exhibit effects similar to those of L-glutamic acid and L-aspartic acid (15). Glutamate is the most prominent and excitatory neurotransmitter at the neuromuscular junction. Glutamate receptors can be subdivided into two classes: ionotropic and metabotropic. Ionotropic glutamate receptors are ligand-gated, nonselective cation channels that allow the flow of Na+, K+, and Ca2+ in response to glutamate binding.

* This work was supported by Grant 2010-0011053 from the National Research Foundation of Korea, funded by the Korean Government. We declare that there are no conflicts of interest.

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‡ The abbreviations used are: AMPK, AMP-activated protein kinase; ACC, acetyl-CoA carboxylase; ANOVA, analysis of variance; CaMKK, calmodulin-dependent protein kinase kinase; CNOX, 6-cyano-7-nitroquinoxaline-2,3-dione; HCSA, homocysteine sulfinic acid; MCPG, α-methyl-4-carboxyphenylglycine; mGluR, metabotropic glutamate receptor; MAPK, mitogen-activated protein kinase; PKCζ, protein kinase C ζ; 2-DOG, 2-deoxyglucose.
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whereas the metabotropic glutamate receptors (mGluRs) are G protein-coupled receptors that modulate a variety of second messenger signaling pathways. Glutamate binding to mGluRs causes G proteins to bind to the intracellular region, thereby affecting multiple pathways. There are many subtypes of glutamate receptors, and it is customary to refer to primary subtypes as chemicals that bind more selectively to the glutamate receptor than glutamate. The distribution of glutamate receptors is not restricted to the neuron, and glutamate receptors are present in a variety of peripheral tissues, including the pancreas (16), testis (17), stroma (18), skeletal muscle tissue (19), and melanocytes (20). The effects of glutamate receptors outside of the nervous system, for example in taste (21), cardiomyocytes (22), pancreas (23), and skin (24), are well known. However, the role of mGluRs in skeletal muscle is unclear.

Diabetes may be associated with dysfunction of glutamate receptors in areas of the central nervous system such as the hippocampus (25). Even though the exact biochemical mechanisms underlying alterations in glutamate receptors are a matter of debate, a link between diabetes and glutamate receptors in the central nervous system is highly likely. In contrast, no molecular mechanism of diabetes has been linked to glutamate receptors in peripheral tissues such as skeletal muscle cells. In this study, to understand the metabolic role of HCSA we determined the effects of the glutamate receptor agonist HCSA on glucose uptake in skeletal muscle cells. We found that HCSA activated glucose uptake in muscle cells and that AMPK and p38 MAPK are involved, providing novel insight into the manner in which HCSA contributes to the metabolic function of skeletal muscles.

EXPERIMENTAL PROCEDURES

Reagents—The following primary antibodies were used: phospho-ACC (Ser79), phospho-AMPK (Thr172), phospho-p38 MAPK, AMPKa2, and p38 MAPK (Cell Signaling Technology, New England Biolabs, Beverly, MA); acetyl-CoA carboxylase (ACC) (Upstate, Billerica, MA); PKCζ and GAPDH (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); and phospho-PKCζ (Epitome Co., Burlingame, CA). Horseradish peroxidase-conjugated secondary antibodies were purchased from Kirkegaard and Perry Laboratories (Gaithersburg, MD). mGluR5 antibody was provided by Dr. C. H. Kim (Yonsei University, Seoul, Korea) (26). Glutamate and dihydroxyphenylglycine were purchased from Sigma-Aldrich. HCSA, insulin, compound C, 5-aminomidazole-4-carboxy-amide-1-d-ribofuranoside (AICAR), STO-609, S(+)-α-amino-4-carboxy-α-methyl-benzeneacetic acid (MCPG), CNQX, and SB203580 were obtained from Calbiochem.

Cell Culture and Animal—L6 muscle cells stably expressing Myc-tagged GLUT4 (L6-GLUT4-Myc) were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum (FBS) and antibiotics at 37 °C in 5% CO2. Cells were grown in culture medium consisting of 500 μl of DMEM containing 0.584 g/liter L-glutamate and 4.5 g/liter glucose mixed with 500 ml of F12 medium containing 0.146 g/liter L-glutamate, 1.8 g/liter glucose, 100 μg/ml gentamicin, 2.5 g/liter sodium carbonate, and 10% heat-inactivated FBS. Mouse myoblast C2C12 cells were maintained in DMEM supplemented with 10% FBS and antibiotics at 37 °C in 5% CO2. Mouse neuroblastoma Neuro2a cells were cultured in minimal essential medium (MEM) supplemented with 10% FBS. BalbC male mice weighing 18–20 g served as experimental subjects.

Plasmid DNA Construct—The full-length cDNA of rat AMPKα2 (kindly provided by Dr. Morris J. Birnbaum, University of Pennsylvania, Philadelphia) was subcloned into the pFLAG-CMV-2 expression vector (Sigma-Aldrich). The K45R (ATP binding site) mutant of AMPKα2 was obtained using site-directed mutagenesis. From the PCR product, the hindIII/smal fragment was also subcloned into the p-FLAG-CMV-2 mammalian expression vector.

Immunoblot Analysis—Cells were grown on 24-well plates and then serum-starved for 36 h prior to treatment with the indicated agents. Following treatment, media were aspirated, and cells were washed twice in ice-cold phosphate-buffered saline (PBS) and lysed in 100 μl of lysis buffer. Samples were then briefly sonicated, heated for 5 min at 95 °C, and centrifuged for 5 min. Supernatants were electrophoresed on 8% SDS-PAGE and transferred to polyvinylidene difluoride membranes. Blots were incubated with primary antibodies overnight at room temperature and then washed six times in Tris-buffered saline/0.1% Tween 20 prior to 1 h of probing with horseradish peroxidase-conjugated secondary antibodies at room temperature. Blots were then visualized via ECL (Amersham Biosciences). In some cases, the blots were stripped and reprobed using other antibodies.

2-Deoxyglucose Uptake—The uptake of 2-deoxyglucose by differentiated C2C12 muscle cells was evaluated. Two days after the myoblasts achieved confluence, differentiation to myotubes was induced by incubation for 6–7 days in α-MEM supplemented with 2% FBS, which was changed every 2 days. Next, cells were washed twice in PBS containing 2.5 mM MgCl2, 1 mM CaCl2, and 20 mM HEPES (pH 7.4) and then incubated with the test compounds in the same buffer at 37 °C. The transport assay was initiated by the addition of 2-deoxy-D-[U-14C]glucose (25 μM; 10 mCi/ml) to each of the wells, and the mixture was incubated for 10 min at 37 °C. The assay was terminated by subsequent washing of the cells with ice-cold PBS. Cells were lysed in 10% SDS or 50 mM NaOH. A scintillation counter was used to determine the radioactivity in lysates extracted in SDS, whereas the total protein content in lysates extracted in NaOH was determined by the Bradford procedure (Bio-Rad Laboratories). Glucose uptake values were corrected for non-carrier-mediated transport by measurement of glucose uptake in the presence of 10 μM cytochalasin B.

Semi-quantitative Reverse Transcription-Polymerase Chain Reaction (RT-PCR)—First strand cDNA synthesis was performed using 1 μg of total RNA, which was isolated from frozen tissues at 55 °C for 20 min using a Thermoscript II one-step RT-PCR kit (Invitrogen). Amplification of cDNA was carried out in the same tube using the GeneAmp System 9700 thermocycler (Applied Biosystems, Warrington, UK). Heating at 94 °C for 5 min inactivated the reverse transcriptase. PCR primers were designed with the following sequences of the mGluR5 primer: forward 5’-TGGGACAATGGGGAATTAAA-3’ and
reverse 5’-TCATGGCTGGAGAGACC-3’. The following PCR conditions were used: 27 cycles for 30 s at 94 °C, 30 s at 56 °C, and 30 s at 72 °C followed by 7 min at 72 °C. The number of PCR cycles used was optimized to ensure amplification in the exponential phase. A 10-μl sample from each RT-PCR product was removed and analyzed by agarose gel electrophoresis. Bands were stained with ethidium bromide and visualized under ultraviolet light. Band intensity was quantified by a gel documentation system (GeneGenius, Syngene, Cambridge, UK).

**Silencing of AMPKα2, mGlurR5, CaMKK, p38 MAPK, and PKCζ**—Mouse skeletal muscle C2C12 cells were seeded in 6-well plates and allowed to grow to 70% confluence for 24 h. Transient transfections were performed with a transfection reagent (Lipofectamine 2000, Invitrogen) according to the manufacturer’s protocol. Briefly, either AMPKα2 (UUCUUAGAGUGUUAUCAA, CGCGAGAGUCUGU-UCC, and GGUCGAGAAUUCAGUCAAA; Dharmacon) or mGlurR5 (AAAGAGAAGUGUACGAGGAA, AAGAUAUGUCAGCGAAGGA, GUGACAGAGACUUCAUA, and GGGUCUAGCAGGG-GAAUUA; Dharmacon) nontargeted control siRNAs were designed (Bioneer Corp., Daejon, Korea). We used CaMKKβ siRNA (GGUGCGAUAUCAGUCAAA, GGACUCUCAUCCUUAAGUA, GAACGAAUCUGUGUUGC, and UCAC-ACCAGUCUCAUAUA; Dharmacon), p38 MAPK (AAGCAGCGAUGUAGGU and AAGACCGAAGACGU-ACUAGG (27), and siRNA PKCζ (GAACUGGUGAGAGGAC- CUU, GGAAACUGAAUAUCUAA, GCUGAAGUGGU-UGCUG, and CGAUGCAGGACAGACCUU (28)). Five μl of siRNA and 5 μl of transfection reagent were first diluted with 95 μl of serum-free medium each and then mixed. The mixtures were then allowed to incubate for 10 min at room temperature and added dropwise to each culture well containing 800 μl of serum-free medium. Four hours after transfection, the medium was exchanged with fresh complete medium. Cells were cultivated for 48 h and lysed.

**Calcium Measurement of Cy3-conjugated mGlurR5 siRNA**—Cy3-mGlurR5 siRNA was transfected to C2C12 muscle cells seeded on chamber slides. Forty-eight hours after transfection, cells were treated with HCSA. For the intracellular calcium image, cells were loaded with fluo-3 AM (1 mg/ml; Molecular Probes, Eugene, OR) for 30 min in a 5% CO2 incubator at 37 °C. The responsiveness of the intracellular calcium level was monitored under a confocal microscope. Excitation of fluo-3 AM was provided by the 488-nm line of an argon laser, and the emission range was 515 nm. The confocal pinhole was set to the manufacturer’s protocol. Briefly, either AMPKα2 (UUCUUAGAGUGUUAUCAA, CGCGAGAGUCUGU-UCC, and GGUCGAGAAUUCAGUCAAA; Dharmacon) or mGlurR5 (AAAGAGAAGUGUACGAGGAA, AAGAUAUGUCAGCGAAGGA, GUGACAGAGACUUCAUA, and GGGUCUAGCAGGG-GAAUUA; Dharmacon) nontargeted control siRNAs were designed (Bioneer Corp., Daejon, Korea). We used CaMKKβ siRNA (GGUGCGAUAUCAGUCAAA, GGACUCUCAUCCUUAAGUA, GAACGAAUCUGUGUUGC, and UCAC-ACCAGUCUCAUAUA; Dharmacon), p38 MAPK (AAGCAGCGAUGUAGGU and AAGACCGAAGACGU-ACUAGG (27), and siRNA PKCζ (GAACUGGUGAGAGGAC- CUU, GGAAACUGAAUAUCUAA, GCUGAAGUGGU-UGCUG, and CGAUGCAGGACAGACCUU (28)). Five μl of siRNA and 5 μl of transfection reagent were first diluted with 95 μl of serum-free medium each and then mixed. The mixtures were then allowed to incubate for 10 min at room temperature and added dropwise to each culture well containing 800 μl of serum-free medium. Four hours after transfection, the medium was exchanged with fresh complete medium. Cells were cultivated for 48 h and lysed.

**Calcium Measurement of Fura-2**—Calcium increases were measured using fura-2-acetoxyethyl ester. Cells were incubated in serum-free DMEM with 4 μM fura-2 at 37 °C for 30 min with continuous stirring. After washing with serum-free DMEM, cells were suspended in serum-free DMEM containing 250 mM sulfipyrazole to prevent dye leakage. Approximately 2 × 10⁶ cells were suspended in calcium-free Ringer-Locke solution (158.4 mM NaCl, 1.2 mM MgCl₂, 5 mM HEPES, 10 mM glucose, and 0.2 mM EGTA (pH 7.3)) for each measurement. Changes in the fluorescence ratio were measured at an emission wavelength of 500 nm for a dual excitation wavelength of 340 and 380 nm. Calibration of the fluorescence ratio versus calcium concentration was performed as described previously (29).

**Determination of the Proportion of GLUT4-Myc at the Cell Surface**—Cell surface GLUT4-Myc was quantified using an antibody-coupled colorimetric assay following treatment with insulin or HCSA as described previously (30). Briefly, to label cell surface GLUT4-Myc in intact myoblasts, cells were exposed to polyclonal anti-Myc antibody (1:100) for 1 h at 4 °C, fixed with 4% paraformaldehyde for 30 min, and incubated with peroxidase-conjugated goat anti-rabbit IgG (1:1000) for 1 h. Cell surface GLUT4-bound anti-Myc antibodies were probed with HRP-conjugated secondary antibodies. Cells were then washed six times and incubated in 1 ml of OPD reagent (0.4 mg/ml o-phenylene diamine dihydrochloride) for 30 min. The optical absorbance of the supernatant was measured at 492 nm.

**Immunofluorescence Measurement of GLUT4-Myc Translocation**—Briefly, L6-GLUT4-Myc-tagged myoblasts were split onto collagen-coated glass coverslips. After 24 h, PKCζ siRNA, which was conjugated with fluorescein isothiocyanate (Bioneeer), was transfected to cells seeded on chamber slides. Forty-eight hours after transfection, cells were serum-starved for 2 h, stimulated with 10 μM HCSA for 30 min at 37 °C, and then fixed with 4% paraformaldehyde in PBS for 20 min at 4 °C. Detection of GLUT4-Myc, cells were incubated for 3 h with primary c-Myc antibody at a dilution of 1:100 in buffer (5% BSA in PBS) at 4 °C followed by incubation with Cy3-conjugated secondary antibody at a dilution of 1:250 for 1 h at room temperature. Cells were observed using a Zeiss LSM510 confocal fluorescence microscope.

**Data Analysis**—Data are expressed as mean ± S.E. Image Gauge (version 3.1; Fujifilm, Tokyo) was used for analysis of band intensity. One-way ANOVA was used followed by a Holm-Sidak multiple range test for comparison between groups. A p value < 0.05 was considered significant.

**RESULTS**

**HCSA Stimulates Glucose Uptake in Differentiated Mouse Myoblast C2C12 Cells**—To elucidate the role of the glutamate receptor agonist HCSA in glucose metabolism, we first examined the effect of HCSA on glucose uptake in differentiated myoblast C2C12 cells. As shown in Fig. 1, 10 μM HCSA increased 2-deoxyglucose uptake in differentiated C2C12 myoblast cells. However, this concentration of HCSA did not influence the viability of C2C12 cells, as assessed by 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; data not shown). Fig. 1 also shows that 100 nM insulin increased glucose uptake, which served as a positive control. To gain insight into the role of the glutamate receptor, we determined the effect of glutamate on glucose uptake and found that glutamate (100 μM) also stimulated 2-deoxyglucose uptake, thus indicating that HCSA may have a metabolic role in skeletal muscle cells.
HCSA Increases Phosphorylation of AMPK in C2C12 Cells—
To characterize the molecular mechanisms of HCSA action, we evaluated its effects on the phosphorylation of AMPK, one of the key metabolic sensor kinases. Administration of HCSA induced a time-dependent increase in AMPK phosphorylation in C2C12 cells (Fig. 2A). As seen in Fig. 2A, the level of phosphorylation of Thr172, which is in the active site of the AMPK-α subunit and essential for enzyme activity, reached a maximum at 1 h and then decreased to the basal level at 3 h. Consistent with the increase in AMPK phosphorylation, the phosphorylation of ACC-Ser79, which is the best characterized site of phosphorylation by AMPK, increased after HCSA administration. HCSA also stimulated AMPK phosphorylation in a dose-dependent manner, with the maximal increase occurring at a concentration of 10 μM (Fig. 2B). Next, RT-PCR and Western blot analysis were performed for the examination of mGluR5 expression in skeletal muscle cells. RT-PCR analysis revealed highly expressed mRNA mGluR5 in C2C12 cells (Fig. 2C). In agreement with RT-PCR findings, Western blot detected protein mGluR5 in cells (Fig. 2D); specifically, mGluR5 appeared as a major band of ~140 kDa. Neuro2a neuron cells were used as a positive control for mGluR5 expression. To support its physiological relevance, we examined the question of whether mGluR5 is expressed in mouse muscle tissue. Protein expression of mGluR5 was clearly detected in mouse quadriceps muscle tissue as well as skeletal muscle C2C12 cells (Fig. 2E). Also, administration of glutamate induced a time- and dose-dependent (Fig. 2, F and G, respectively) increase in AMPK phosphorylation. These results indicate that HCSA may have a metabolic role in skeletal muscle cells through the glutamate receptor-mediated signal pathway.

Intracellular Calcium Is Involved in HCSA-induced AMPK Phosphorylation—For elucidation of the signal pathway underlying HCSA-induced AMPK phosphorylation, we investigated the intracellular calcium release using fura-2 after HCSA treatment. Fig. 3A shows that the intracellular calcium concentration increased from a concentration of 50 nM to a concentration of 150 nM following treatment of C2C12 cells with HCSA. To confirm the involvement of the glutamate receptor, we used the mGluR5 antagonist MCPG. Pretreatment of cells with MCPG significantly blocked the HCSA-induced increase in intracellular calcium concentration (Fig. 3A). However, the HCSA-induced increase in intracellular calcium concentration persisted in the presence of CNQX, an AMPA antagonist, suggesting the involvement of mGluR in HCSA-induced calcium signaling. To correlate the role of calcium in HCSA-induced AMPK, we assessed the effect of the above antagonists. The HCSA-induced increase in AMPK/ACC phosphorylation disappeared in the presence of MCPG but not CNQX (Fig. 3B). Similarly, MCPG pretreatment blocked HCSA-induced glucose uptake (Fig. 3C). To confirm the role of mGluR5 in the calcium response at the single cell level, we constructed Cy3-conjugated mGluR5 siRNA. As shown in Fig. 3D, the increase in the intracellular calcium concentration was significantly suppressed by knockdown of mGluR5 by transfection with Cy3-conjugated mGluR5 siRNA. Moreover, phosphorylation of AMPK did not occur following knockdown with siRNA mGluR5 (Fig. 3E). Accordingly, the increase in HCSA-induced glucose uptake was not observed following mGluR5 knockdown (Fig. 3F). The HCSA-induced increase in AMPK phosphorylation disappeared in the presence of STO-609, a CaMKK inhibitor (Fig. 3G), suggesting involvement of calcium in HCSA-mediated AMPK phosphorylation. To further confirm involvement of calcium, we silenced CaMKK. Fig. 3H shows that HCSA-induced AMPK phosphorylation was suppressed by knockdown of CaMKK. To provide direct evidence of the role of the mGluR receptor, we used a specific mGluR receptor agonist, dihydroxyphenylglycine, and as shown in Fig. 3I, phosphorylation of AMPK was increased by treatment of cells with dihydroxyphenylglycine, implicating this receptor in AMPK signaling. Collectively, these results suggest that HCSA induces AMPK phosphorylation through intracellular calcium.

HCSA Activates the p38 MAPK Pathway in an AMPK-dependent Fashion—To further elucidate the signal pathways involved in HCSA-mediated glucose uptake, we investigated the effects of HCSA on p38 MAPK. HCSA (10 μM) was shown to phosphorylate p38 MAPK (Fig. 4A). To determine whether AMPK is involved in the effect of HCSA, we investigated p38 MAPK phosphorylation following AMPKa2 dominant-negative expression. As shown in Fig. 4B, the effect of HCSA on p38 MAPK phosphorylation was completely blocked in C2C12 cells that had been transiently transfected with dominant-negative AMPKa2, indicating the involvement of AMPK in HCSA-induced p38 MAPK signaling. Together, these results indicate that p38 MAPK operates in the HCSA-mediated signaling pathway in an AMPK-dependent manner.

Inhibition of AMPK and p38 MAPK Blocks HCSA-mediated Glucose Uptake—To corroborate the roles of AMPK and p38 MAPK in HCSA-mediated glucose uptake, we assessed the effect of inhibitors of these kinases. To this end, C2C12 cells were pretreated with compound C, an AMPK inhibitor,
SB203580, or a p38 MAPK inhibitor in the presence of HCSCA (10 μM). The effect of HCSCA on glucose uptake was attenuated in C2C12 cells that had been treated with inhibitors (Fig. 5), indicating the involvement of these pathways in HCSCA-induced glucose uptake. Therefore, these findings show that HCSCA increases the activities of AMPK and p38 MAPK, eventually leading to stimulation of glucose uptake.

HCSCA Stimulates PKCζ through the p38 MAPK Pathway—To explain the mechanism of HCSCA-mediated glucose uptake, we examined the effect of HCSCA on PKCζ, a convergent downstream molecule for glucose uptake. Fig. 6A shows that HCSCA increased phosphorylation of PKCζ in a time-dependent fashion. Next, we examined PKCζ phosphorylation following AMPKα2 knockdown and found that HCSCA-mediated phosphorylation of PKCζ was suppressed following AMPKα2 knockdown (Fig. 6B). To determine the hierarchy between PKCζ and p38 MAPK, we assessed the effect of p38 MAPK knockdown on PKCζ phosphorylation and observed that knockdown of p38 MAPK blocked HCSCA-induced PKCζ phosphorylation, indicating that p38 MAPK plays a role as a PKCζ upstream (Fig. 6C). Pharmacologic inhibition of p38 MAPK with SB203580 also suppressed HCSCA-induced PKCζ phosphorylation (Fig. 6D). Together, these results indicate an important role for PKCζ in HCSCA-induced signaling transduction in downstream p38 MAPK.

PKCζ Is Involved in HCSCA-induced GLUT4 Translation—To provide physiological relevance, the levels of GLUT4-Myc present in the plasma membrane were measured in L6 myo-
blasts that stably express GLUT4-Myc (L6-GLUT4-Myc). This cell line was used to establish a single cell assay for GLUT4 translocation. The Myc epitope is on the first extracellular segment of GLUT4, facilitating recognition of cell surface transporters by extracellular labeling without the need to perform subcellular fractionation. First, we found that 300 nM siRNA PKC\(_{\text{H9256}}\) silenced PKC\(_{\text{H9256}}\) expression (Fig. 7A).

We then measured the amount of GLUT4-Myc at the plasma membrane. Knockdown of PKC\(_{\text{H9256}}\) inhibited the HCSA-induced increase in the levels of GLUT4-Myc at the plasma membrane (Fig. 7B), suggesting the importance of PKC\(_{\text{H9256}}\) for glucose uptake. To confirm the effect of PKC\(_{\text{H9256}}\) knockdown on the translocation of GLUT4-Myc to the cell surface at the single cell level, we constructed FITC-conjugated PKC\(_{\text{H9256}}\).
siRNA to facilitate recognition of transfected cells (PKCζ siRNA-transfected cells are shown in blue (Fig. 7C). HCSA caused an increase in surface-exposed Myc epitope (Fig. 7C, left panel). However, transfection of FITC-conjugated PKCζ siRNA impaired cell surface GLUT4-Myc translocation levels (Fig. 7C, right panel). PKCζ knockdown confined major portions of GLUT4 to the perinuclear regions. These results further indicate that PKCζ plays a critical role in HCSA-induced glucose uptake.

DISCUSSION

The principal finding of this study was that HCSA, a glutamate receptor agonist, has a metabolic function in skeletal systems. Specifically, we demonstrated that AMPK and p38 MAPK are instrumental in HCSA-mediated glucose regulation.

The role of HCSA had been evaluated previously in conjunction with cardiovascular and neuronal diseases (2–6). The glutamate receptor agonistic properties of HCSA appear to be responsible for the risk for atherosclerosis and may also contribute to the observed neuropathologic roles (4–6). However, the metabolic function of HCSA has not been adequately elucidated. In the present study, we established that HCSA increased glucose uptake through the AMPK pathway. In addition, HCSA also activated p38 MAPK, a critical regulator of glucose uptake. Collectively, our results suggest that both AMPK and p38 MAPK play crucial roles in HCSA-mediated glucose metabolism. The contribution of HCSA to metabolic function has raised several questions as to which of the characteristics of HCSA are relevant for its participation in metabolic function. HCSA levels in normal human serum are in the nanomolar range (31). In the present study, we showed that an HCSA concentration of 10 μM affected the intracellular calcium concentration and AMPK phosphorylation, indicating that somewhat higher concentrations of HCSA are required to effectively determine its effect in a cell culture system, even though the physiologically relevant concentration is low by comparison. There are two probable reasons for this disparity. First, the in vitro and in vivo environments are different. Second, the microenvironment of an immortalized skeletal muscle cell line may differ from that of normal muscles. It has been established that moderate hyperhomocysteinemia (15–30 μM) is prevalent in individuals with type 2 diabetes. Concentrations of homocysteine in severe cases may reach almost millimolar ranges. Acidic oxidized derivatives of homocysteine, including HCSA, are potent and selective agonists of mGluR. One radioligand binding study has demonstrated that HCSA has high binding affinity for mGluR (32). In addition, the effective concentrations (EC50) of HCSA and glutamate are about 5 and 9 μM, respectively (33), indicating that HCSA is as potent as glutamate, a well characterized endogenous neu-

![FIGURE 4. HCSA activates the p38 MAPK pathway in an AMPK-dependent fashion. A, C2C12 cells were stimulated with HCSA (10 μM) for the indicated times. Cell lysates were analyzed by Western blotting (IB) using anti-phospho-p38 MAPK antibody. Blotting with anti-p38 MAPK antibody was conducted as a protein loading control. B, C2C12 cells were transiently transfected with dominant-negative AMPKα2 for 48 h. Following starvation, cells were stimulated with HCSA (10 μM) for 1 h. Cell lysates were analyzed by Western blotting using anti-phospho-p38 MAPK antibody. Blotting with anti-p38 MAPK was conducted as protein loading control. A and B, data in the bar graphs represent the mean ± S.E. values of the ratios of densities (p-p38 MAPK/p38 MAPK) for at least three independent Western blot experiments. *, p < 0.05 versus basal values.](http://www.jbc.org/)

![FIGURE 5. Inhibition of AMPK and p38 MAPK inhibits HCSA-mediated glucose uptake. Differentiated C2C12 cells were incubated in 60-mm dishes for 1 h with either HCSA (10 μM) or insulin (100 nM) alone in the presence of either compound C or SB203580 and then assayed for 2-DG uptake as described under “Experimental Procedures.” *, p < 0.05, as compared with the insulin alone sample values (one-way ANOVA and Holm-Sidak comparisons). Each value is expressed as the mean ± S.E. of four determinations.](http://www.jbc.org/)

[Effect of Homocysteine Sulfonic Acid on AMPK](http://www.jbc.org/)
rotransmitter for mGluR. Several studies have suggested the involvement of mGluR in diabetes. In diabetes animal models, the expression of mGluR is increased in nervous tissue (34, 35), suggesting that mGluR may be associated with the pathogenesis of diabetes. mGluR is also expressed in pancreatic islet cells (16) and can have an impact upon the release of glucagon release. Because our experimental studies were conducted under nonphysiological conditions, the potential clinical implications of HCSA are not clarified as yet. The source of endogenous HCSA is methionine cycled through the transmethylation pathway (36). Therefore it is conceivable that the concentration of HCSA is elevated in serum under conditions of hyperhomocysteinemia, and thus it may be implicated in the pathogenesis of diabetes. Recent studies have revealed that mGluR is expressed in the non-neuronal system. Herein, we further demonstrated that HCSA increases the phosphorylation of AMPK in skeletal muscle cells. Taken together, the biological significance of our study is based on the demonstration that a sufficient elevation in local concentrations of HCSA is present in vivo, particularly in diseases associated with hyperhomocysteinemia, such as diabetes. Further study should be focused on the mechanism by which HCSA may act on diabetes.

The objective of the present study was to ascertain whether the glucose metabolism of skeletal muscles is regulated directly by HCSA and, if so, to determine which molecules are involved in this process. Our data show that HCSA increased the intracellular calcium concentration in skeletal muscle cells, implying that HCSA may directly stimulate AMPK through a glutamate receptor-mediated increase in calcium. Because CaMKK is known to be the upstream kinase of AMPK (37, 38), the present results suggest that the effect of HCSA-induced AMPK phosphorylation in skeletal muscle cells is probably mediated by CaMKK. Even though the mechanism by which glutamate is involved in metabolism remains unknown, the present results strongly indicate that HCSA increases glucose uptake through the calcium-induced AMPK-p38 MAPK pathway. Furthermore, the peripheral distribution of the glutamate receptor suggests the existence of a non-neuronal function of glutamate signaling, and a recent study has demonstrated a peripheral function of glutamate in skeletal muscles, such as the synap-
tic assembly in the neuromuscular junction (39). The results described herein provide the first evidence of the association of glutamate-mediated signaling with the regulation of glucose homeostasis in the skeletal muscle system.

Hyperhomocysteinemia is common in diabetic patients, suggesting a possible relationship between homocysteine and diabetic pathogenesis. At the same time, recent evidence indicates the presence of glutamate receptors in non-neuronal tissues, such as skeletal muscle tissue, with functional implications for the involvement of homocysteine in peripheral skeletal muscle tissues. The key finding of this study is that HCSA, as an mGluR agonist, may offer an exciting prospect for the management of diabetes because it triggers AMPK phosphorylation through mGluR receptor-mediated calcium signaling. In the present study, we have highlighted the potential clinical application of HCSA in diabetes through regulation of the metabotropic glutamate system; this may be vital for the clinical utility of HCSA.

In conclusion, HCSA activates AMPK and p38 MAPK in skeletal muscle cells, and intracellular calcium exerts a profound influence on HCSA-AMPK phosphorylation. Future studies should focus on elucidating the relationship between AMPK and glutamate receptor-mediated calcium within the context of the HCSA-mediated signaling pathway. We are currently in the process of further investigating this relationship.

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