6-Mercaptopurine augments glucose transport activity in skeletal muscle cells in part via a mechanism dependent upon orphan nuclear receptor NR4A3

Qinglan Liu,1 Xiaolin Zhu,2 Lusheng Xu,2 Yuchang Fu,2 and W. Timothy Garvey2,3

1Department of Pathology, University of Alabama at Birmingham, Birmingham, Alabama; 2Department of Nutrition Sciences, University of Alabama at Birmingham, Birmingham, Alabama; and 3Birmingham Veterans Affairs Medical Center, Birmingham, Alabama

Submitted 25 March 2013; accepted in final form 29 August 2013

Liu Q, Zhu X, Xu L, Fu Y, Garvey WT. 6-Mercaptopurine augments glucose transport activity in skeletal muscle cells in part via a mechanism dependent upon orphan nuclear receptor NR4A3. Am J Physiol Endocrinol Metab 305: E1081–E1092, 2013. First published September 6, 2013; doi:10.1152/ajpendo.00169.2013.—The purine anti-metabolite 6-mercaptopurine (6-MP) is widely used for the treatment of leukemia and inflammatory diseases. The cellular effects of 6-MP on metabolism remain unknown; however, 6-MP was recently found to activate the orphan nuclear receptor NR4A3 in skeletal muscle cell lines. We have reported previously that NR4A3 (also known as NOR-1, MINOR) is a positive regulator of insulin sensitivity in adipocytes. To further explore the role of NR4A3 activation in insulin action, we explored whether 6-MP activation of NR4A3 could modulate glucose transport system activity in L6 skeletal muscle cells. We found that 6-MP increased both NR4A3 expression and NR4A3 transcriptional activity and enhanced glucose transport activity via increasing GLUT4 translocation in both basal and insulin-stimulated L6 cells in an NR4A3-dependent manner. Furthermore, 6-MP increased levels of phospho-AS160, although this effect was not modulated by NR4A3 overexpression or knockdown. These primary findings provide a novel proof of principle that 6-MP, a small molecule NR4A3 agonist, can augment glucose uptake in insulin target cells, although this occurs via both NR4A3-dependent and -independent actions; the latter is related to an increase in phospho-AS160. These results establish a novel target for development of new treatments for insulin resistance.

NR4A3; 6-mercaptopurine; glucose transport; glucose transporter 4 translocation; Akt substrate of 160 kDa; insulin action; skeletal muscle

INSULIN RESISTANCE IS CENTRAL to the pathogenesis of metabolic syndrome, prediabetes, type 2 diabetes, and cardiovascular disease risk (5, 31) and involves diminished stimulation of glucose transport in skeletal muscle (11). Thus, improving insulin sensitivity, whether achieved via lifestyle or pharmacoceutical interventions, is essential for both prevention and treatment of type 2 diabetes and other metabolic disorders (16). Currently, thiazolidinediones are the only class of antidiabetic drugs that act primarily by increasing insulin sensitivity in muscle and establish a precedent for the use of transcription factor agonists (i.e., PPARγ) as insulin-sensitizing agents (4). However, thiazolidinediones can cause unwanted side effects such as weight gain, edema, and increased risk of bone fractures (20, 24). The limitation of current medications highlights the need for finding alternative molecular regulators in insulin action and developing new insulin sensitizers.

To find new molecular regulators, we have employed cDNA microarrays to examine differential gene expression in skeletal muscle biopsies from insulin-sensitive and -resistant human subjects (39). We have found that the orphan nuclear receptor NR4A3 (also known as NOR-1, MINOR) can be induced by insulin during hyperinsulinemic euglycemic clamp studies and was reduced in skeletal muscle of insulin-resistant subjects (39) as well as in muscle and adipose tissue from rodent models of insulin resistance and diabetes (10). NR4A3 is one of three members of the nuclear receptor family NR4A, which is a constituent of the nuclear hormone receptor superfamily, a large group of transcription factors regulating multiple biological roles, including metabolism and metabolic diseases (13, 35, 42). Because activating ligands have not been identified, NR4As (NR4A1, NR4A2, and NR4A3) are termed as orphan nuclear receptors. NR4A family members share homology in their basic structures (35). They are early-response genes that can respond to diverse stimuli such as fatty acids, growth factors, and inflammatory cytokines and mediate several functional responses involved in neural differentiation, mitogenesis, apoptosis, inflammation, and metabolism in a tissue-specific manner (21). NR4A3 expression is restricted to a few tissues, including peripheral insulin target tissues (muscle and fat) in humans, whereas NR4A1 (also known as Nur77) is more ubiquitously expressed, and little or no NR4A2 is expressed in muscle or fat (23, 25).

In addition to our observation that skeletal muscle NR4A3 expression is reduced in insulin-resistant humans and rodents, we have found that NR4A3 overexpression in adipocytes increases and that shRNA knockdown suppresses insulin-responsive glucose transport and GLUT4 translocation as well as insulin-mediated insulin receptor substrate-1 (IRS-1) and Akt phosphorylation (10). These findings indicate that NR4A3 positively modulates insulin sensitivity and glucose uptake, making it an attractive target for treating insulin resistance. Therefore, we sought to identify agonists of NR4A3 that can increase insulin sensitivity, especially in skeletal muscle, which is responsible for the bulk of insulin-stimulated glucose uptake in humans (30). Although no endogenous ligands have been found for NR4A family, structure studies revealed that the ligand-binding domains (LBDs) of all three NR4A members lack a conventional ligand-binding cavity due to bulky hydrophobic residues (3) but they contain a hydrophobic surface in the ligand-binding domain that interacts with cofactors modulating transcriptional activity (8). Recently, it was reported that 6-mercaptopurine (6-MP) specifically transactivates NR4A3 in C2C12 cells by interacting with the AF-1 domain involved in coactivator recruitment (37, 38). 6-MP belongs to the thiopurine chemical family, a group of compounds struc-
6-MERCAPTOPURINE ACTIVATES GLUCOSE TRANSPORT VIA NR4A3

**MATERIALS AND METHODS**

**Reagents.** Chemicals were purchased from Sigma (St. Louis, MO) unless otherwise indicated. Cell culture medium and serum were purchased from Invitrogen. 2-deoxy-D-[3H]glucose was purchased from Perkin-Elmer Life and Analytical Science. Anti-NR4A3 antibody (Rabbit pAb, IMG-79195) was purchased from Imgenex (San Diego, CA). Anti-β-actin antibody (Mouse mAb, AC-15) was purchased from Sigma. Anti-Akt substrate of 160 kDa (AS160) antibody (Rabbit pAb, no. 07–741) was purchased from Upstate (Lake Placid, NY). Anti-phospho-AS160 (Thr642, rabbit, no. 441071G) was purchased from Biosource (Camarillo, CA). Anti-GLUT4 (Mouse mAb, 1FB, no. 2213S) was purchased from Cell Signaling Technology (Boston, MA). GLUT1 (sc-7903) and secondary antibodies were purchased from Santa Cruz Biotechnology.

**Cell culture and stimulation.** L6 rat skeletal muscle cells and NIH3T3 cells (mouse fibroblasts) were obtained from ATCC (Manassas, VA). L6 myoblasts stably expressing GLUT4 with an exonofacial myc epitope (L6-GLUT4myc) were a kind gift from Dr. Amira Klip (University of Toronto). All L6 skeletal muscle cells were grown and maintained as myoblasts in Dulbecco’s minimal essential medium (DMEM) containing 4.5 g/l glucose, 0.1% (v/v) rabbit serum, 100 U/ml penicillin, 100 mg/ml streptomycin, and 10% FBS in a humidified 5% CO2 atmosphere at 37°C. NIH3T3 cells were grown in the same condition. To obtain differentiated L6 myotubes, 70–80% confluent myoblasts were induced by reducing serum concentration to 2% horse serum for 6–10 days before experiments.

**Recombinant lentiviruses and lentiviral-transduced cell lines.** Lentiviral-transduced NR4A3 overexpression has been described previously (10). Briefly, full-length human NR4A3 cDNA coding sequence and a V5 epitope tag were cloned into a Vira-Power-CMV vector (Invitrogen). The recombinant NR4A3 construct or a control LacZ gene plasmid was transfected into human embryonic kidney (HEK)-293 cells. After a successful transfection (confirmed by Western blots), infectious virus particles were generated according to the manufacturer’s protocol (Invitrogen). To establish stably expressing cell lines, recombinant NR4A3 or LacZ lentiviral stocks were used to infect L6 or L6-GLUT4myc cells with Polybrene at a final concentration of 6 μg/ml. Forty-eight hours after infection, cells were placed under puromycin selection (3 μg/ml) for 14 days. Western blot analyses were performed to test for stable NR4A3 or LacZ expression after antibiotic selection.

**For lentiviral based endogenous NR4A3 gene knockdown, three NR4A3 shRNA plasmid DNA constructs (in PLKO.1 lentiviral vector backbone) complementary to rat NR4A3 gene coding sequences were purchased from Sigma (MISSION, no. NM_015734). The shRNA sequences are 5′-CCGCGGCCTTTGTGATCAGATGGAACCTGCAG-3′, 5′-CCGGGGGGAATCCGTAGGATCTGAAAGACTGGGCTTCTTGTTGCAATCCATGGATGACATGAGC-3′, and 5′-CCGGGGGGAATCCGTAGGATCTGAAAGACTGGGCTTCTTGTTGCAATCCATGGATGACATGAGC-3′. The recombinant shRNA-NR4A3 lentiviral plasmid or nontarget shRNA control vector (MISSION, no. SHC002; Sigma) was transfected into HEK-293 cells to generate lentiviruses. Thereafter, shRNA-NR4A3 lentiviruses were transduced into L6 or L6-GLUT4myc myoblasts to establish cell lines hyppressing NR4A3. Stable knockdown cell lines were selected under the same conditions as selecting NR4A3 overexpression cell lines described above.

**Glucose transport activity assay.** Glucose transport rate was assayed in monolayers as initial rates of 2-deoxy-D-[3H]glucose uptake, as described previously (22). Here, L6 myotubes in six-well tissue culture plates were treated with DMSO control or 6-MP for 24 h, with the final 3 h of incubation including treatments in serum-free DMEM. Then the cells were washed three times with glucose-free Krebs-Ringer phosphate HEPES (KRPH) buffer (20 mM HEPES, 120 mM NaCl, 1.2 mM MgSO4, 2 mM CaCl2, 2.5 mM KCl, 1 mM NaH2PO4, and 1 mM sodium pyruvate) with 0.2% BSA and further incubated in the absence or presence of insulin (100 nM) for 60 min at 37°C. 2-deoxy-D-[3H]glucose (0.1 mM, 0.2 μCi/ml) was then added for 3 min for cells to uptake. After that, cells were washed three times with ice-cold KRPH buffer containing Phloretin (300.9 mM) to remove the remaining 2-deoxy-D-[3H]glucose in the buffer. Finally, cells were lysed with 1 M NaOH, and glucose uptake rates were determined by measurement of 3H radioactivity via liquid scintillation counting. Results were normalized by cellular protein content determined by BCA protein assay (no. 23227; Thermo Scientific).

**GLUT4 translocation assay.** The recruitment of intracellular myc-tagged GLUT4 to the cell surface upon NR4A3 agonist and insulin stimulation was measured by an antibody-coupled colorimetric assay (36). L6-GLUT4myc cells cultured in 24-well plates were treated with DMSO control or 6-MP for 24 h, with the final 2 h of incubation including treatments in serum-free DMEM, and further incubated in the absence or presence of insulin 100 nM for 30 min at 37°C. After incubation, cells were quickly washed once with cold PBS and then fixed with 3% paraformaldehyde in PBS for 10 min at 4°C, followed by 20 min at room temperature. Fixative was then neutralized by incubation with 50 mM NH4Cl in PBS at room temperature for 10 min. Next, cells were blocked with 5% nonfat milk in PBS at room temperature for 15 min. Primary mouse monoclonal antibody anti-c-myc (9E10; Santa Cruz Biotechnology) was then added to the cultures at a dilution of 1:200 in 5% goat serum PBS for 60 min at 4°C. Cells were washed extensively with PBS before incubating with 1:1,000 dilution of horseradish peroxidase-conjugated goat anti-mouse IgG in PBS containing 5% goat serum. After 30 min at room temperature the cells were washed extensively, and 1 ml of OPD reagent (0.4 mg/ml o-phenylenediamine dihydrochloride and 0.4 mg/ml urea hydrogen peroxide in H2O, prepared according to the manufacturer’s guidelines) was added to each well at room temperature and incubated for 30 min, protected from light. The reaction was stopped by the addition of 0.25 ml of 3 M HCl. The supernatant was collected, and the optical absorbance (measured at 492 nm) reflected the amount of immunoreactive cell surface GLUT4myc. Intra-assay control wells remained untreated with either primary antibody or both primary and secondary antibodies. Measurements of surface GLUT4myc in control wells were subtracted from values obtained from all other experimental conditions. Results were normalized by cellular protein concentration determined by BCA protein assay.

**NR4A3 transcriptional activity assay.** To assess NR4A3 transcriptional activity, we established a cell-based “one-hybrid” luciferase assay system. Briefly, NR4A3 was fused with the DNA-binding domain of yeast GAL4 transcription factor (GAL4DBD; Promega). Then, this NR4A3-GAL4DBD fusion gene and the luciferase response gene UAS-Luc (luc2P luciferase reporter under the control of 9 repeats of the GAL4 Upstream Activation Sequence; Promega) were cotransfected into the NIH3T3-reporting cell line. Stable cells were further selected for quantification of NR4A3 transcriptional activity. Cells were seeded in 96-well plates and treated with DMSO control or 6-MP for 24 h. Then, NR4A3 transcriptional activity reflected by luciferase response gene expression was measured by luminometry.
Cell viability assay. Cell viability was measured using CellTiter-Glo Luminescent Cell Viability Assay (no. G7570; Promega). L6 skeletal muscle cells were seeded in 96-well plates at a density of 10,000 cells/well and differentiated into myotubes within 7 days. Cells were treated with different doses of 6-MP for 24 h before the assay. For analysis of cell viability, plates were equilibrated at room temperature for 30 min; 50 μl of Cell Titer-Glo reagent was added to each well, and plates were mixed for 12 min on an orbital shaker. Luminescence was quantified using a luminometer.

Western blot analysis. Levels of total and phosphorylated proteins were detected in cell lysates by Western immunoblotting. L6 myotubes were treated with DMSO control or 6-MP for 24 h, with the final 3 h of incubation including treatments in serum-free DMEM, and further incubated in the absence or presence of 100 nM insulin for 60 min at 37°C. Then, protein lysates (50 μg) were collected and subjected to SDS-PAGE and immunoblotted with primary antibodies against NR4A3, GLUT4, AS160, phospho-AS160, IRS-1, phospho-IRS-1, Akt, phospho-Akt, phospho-GSK-3β, phospho-GSK-3β phosphorylated AMP-activated protein kinase-α (AMPKα), AMPKα, β-actin overnight at 4°C. We then used horseradish peroxidase-conjugated secondary antibodies, chemiluminescence, and X-ray films to detect blots. The proteins were finally quantified by densitometric analysis of scanned films using Image J software.

RNA preparation and RT-PCR analysis. Total RNA from L6 myotubes was extracted using RNAeasy Mini Kit (Qiagen no. 74104) and then reverse transcribed to first-strand cDNAs using the Super-Script VILO cDNA Synthesis Kit (no. 11754; Invitrogen). For PCR amplification from cDNAs, 20 μl of reaction mixture contains: 1 μl of the first-strand cDNA template, and 7 μl of H2O. Specific oligonucleotide primers (pur chased from IDT) are as follows: for rat AS160, 5'-ATGGTACGTT-GGAGGCTTTCC-3' (sense) and 5'-GGCTGGGTTTTCTGGTTGTG-TAG-3' (antisense); for rat PPARγ coactivator-1α (PGC-1α), 5'-GGCCGGTACAGTTGAGTGT-3' (sense) and 5'-GCACTGAGGACTTGCTGATT-3' (antisense); for rat 18S, 5'-CGCTTCTTTACTTGTTGAT-3' (sense) and 5'-TCCCCCTCGGAATCGAA-3' (antisense). The thermal cycling program was 3 min at 95°C for enzyme activation; 35–37 cycles of 1 min at 57°C, and 1 min of annealing at 57°C, and 1 min of extension at 72°C; followed by 10 min at 72°C. Total amplified product was loaded in an agarose gel (1%) containing SYBR safe DNA gel stain (Invitrogen), and the electrophoresis was conducted. After gel electrophoresis, DNA bands were visualized by UV transillumination, and the intensity was quantified using Image J software.

EC50 calculation. We calculated EC50 for dose response of 6-MP’s effect on basal and insulin-stimulated glucose transport in L6 myotubes using Microsoft Excel software. First, dose points “x” (0, 5, 10, 25, 50, and 75 μM) were logarithm-transformed to “X” to better fit dose-responsive data to linear regression analysis: X = logx (x = 10^S). Next, we plotted an X-Y scatter graph, with y-axis = glucose transport rates and x-axis = X (log-transformed dose points). Then we conducted linear regression analysis for the graph, and the linear equations for each basal and insulin-stimulated dose data were generated as Y = aX + B. From the linear equations, we calculated EC50 using the following equations: Y50% = (Ymin + Ymax)/2, X50% = (Y50% – B)/a, and EC50 = x50% = 10^X50%.

Statistical analysis. Experimental results are shown as means ± SD. P values were calculated using a two-tailed Student t-test. P < 0.05 was considered significant.

**Fig. 1.** Effects of 6-mercaptopurine (6-MP) on glucose transport activity and cell viability in L6 myotubes. A: dose-responsive effect of 6-MP on glucose transport activity. Fully differentiated L6 rat skeletal muscle cells (L6 myotubes) were treated with DMSO vehicle control (0 μM) or 5–75 μM 6-MP for 24 h (with the final 3 h under serum starvation) and further incubated in the absence (basal) or presence of insulin (100 nM) for 60 min at 37°C. 2-deoxy-D-[3H]glucose was chased from IDT) are as follows: for rat AS160, 5'-ATGGTACGTT-GGAGGCTTTCC-3' (sense) and 5'-GGCTGGGTTTTCTGGTTGTG-TAG-3' (antisense); for rat PPARγ coactivator-1α (PGC-1α), 5'-GGCCGGTACAGTTGAGTGT-3' (sense) and 5'-GCACTGAGGACTTGCTGATT-3' (antisense); for rat 18S, 5'-CGCTTCTTTACTTGTTGAT-3' (sense) and 5'-TCCCCCTCGGAATCGAA-3' (antisense). The thermal cycling program was 3 min at 95°C for enzyme activation; 35–37 cycles of 1 min at 57°C, and 1 min of annealing at 57°C, and 1 min of extension at 72°C; followed by 10 min at 72°C. Total amplified product was loaded in an agarose gel (1%) containing SYBR safe DNA gel stain (Invitrogen), and the electrophoresis was conducted. After gel electrophoresis, DNA bands were visualized by UV transillumination, and the intensity was quantified using Image J software.

EC50 calculation. We calculated EC50 for dose response of 6-MP’s effect on basal and insulin-stimulated glucose transport in L6 myotubes using Microsoft Excel software. First, dose points “x” (0, 5, 10, 25, 50, and 75 μM) were logarithm-transformed to “X” to better fit dose-responsive data to linear regression analysis: X = logx (x = 10^S). Next, we plotted an X-Y scatter graph, with y-axis = glucose transport rates and x-axis = X (log-transformed dose points). Then we conducted linear regression analysis for the graph, and the linear equations for each basal and insulin-stimulated dose data were generated as Y = aX + B. From the linear equations, we calculated EC50 using the following equations: Y50% = (Ymin + Ymax)/2, X50% = (Y50% – B)/a, and EC50 = x50% = 10^X50%.

Statistical analysis. Experimental results are shown as means ± SD. P values were calculated using a two-tailed Student t-test. P < 0.05 was considered significant.
6-MP increases basal and insulin-stimulated glucose transport in L6 myotubes. We first assessed 6-MP’s effects on glucose transport activity in L6 skeletal muscle cells, a myogenic line derived from primary cultures of rat thigh muscle widely employed to study insulin action (39). We treated fully differentiated L6 myotubes with 0–75 μM of 6-MP and then measured basal and insulin-stimulated (100 nM) 2-deoxyglucose transport rates. As shown in Fig. 1A, we found that, with vehicle treatment, insulin was able to induce glucose transport 1.7-fold compared with basal L6 cells and that 6-MP treatment augmented both basal and insulin-stimulated glucose transport activity in a dose-responsive manner. Under basal conditions (i.e., without insulin), 6-MP significantly increased glucose transport rates at all concentrations (5–75 μM) over vehicle-treated controls (1.4- to 1.9-fold, most P < 0.01) with a half-maximally effective concentration (EC50) of 6-MP equal to 7.44 μM. Similarly, 6-MP preincubation significantly increased glucose transport at most 6-MP concentrations, with an EC50 of 7.92 μM under insulin-stimulated conditions. In Fig. 1B, the time course of 6-MP treatment demonstrated augmentation of both basal and insulin-stimulated glucose transport as early as at 6 h, and the uptake rate peaked at ~24 h. In Fig. 1C, we conducted cell viability assay for 6-MP treatments on L6 myotubes at 24 h. These data indicated that 6-MP did not impact cell viability at doses of 10 μM or below, which are doses utilized in our experiments, although we did observe reductions in cell viability at higher concentrations.

6-MP enhances both NR4A3 transcriptional activity and protein expression. Since 6-MP is reported to be an activator of NR4A3 in C2C12 muscle cells (37, 38), we next examined whether NR4A3 transcriptional activity or protein expression was modified by 6-MP in our cell systems. To assess NR4A3 transcriptional activity, we developed a cell-based luciferase assay system in NIH3T3 reporter cells. Cells were stably transfected with NR4A3-GAL4DBD fusion/luciferase reporter constructs and treated with different doses (0–100 μM) of 6-MP, followed by quantification of lucinescence. We observed that 6-MP induced NR4A3 transcriptional activity 1.6- to 11-fold (P < 0.01) in a dose-responsive manner (Fig. 2A). To determine whether 6-MP could also regulate NR4A3 protein expression in skeletal muscle cells, we collected total protein lysates from L6 myotubes treated with 0–75 μM of 6-MP and measured NR4A3 expression by Western blot. We found that 6-MP led to a dose-dependent increase in NR4A3 protein levels (Fig. 2B). Together, these results demonstrate that 6-MP not only enhanced NR4A3 activity as a transcription factor but also increased NR4A3 protein expression.

NR4A3 overexpression increases glucose transport rates and augments 6-MP’s ability to stimulate transport in L6 skeletal muscle cells. To determine whether NR4A3 overexpression affects glucose transport, we stably overexpressed NR4A3 in L6 cells using a lentivirus expression vector. We collected protein lysates from LacZ vector control and NR4A3-overexpressing L6 myocytes treated with or without 10 μM 6-MP under basal and insulin-stimulated conditions and quantified NR4A3 protein levels in these cell lines, as shown in Fig. 3A. Stable transfection with lentivirus-NR4A3 resulted in a 70% increase (P < 0.05) in NR4A3 expression compared with LacZ controls. 6-MP treatment both augmented NR4A3 in the control cells and further increased NR4A3 levels in the overexpressing cells (P < 0.05; Fig. 3A). We then measured effects on glucose transport activity and observed that both NR4A3 overexpression and 6-MP led to increased transport rates (Fig. 3B). NR4A3 overexpression alone increased glucose transport activity as compared with LacZ controls.

![Figure 2](http://ajpendo.physiology.org/)

**Fig. 2.** Effects of 6-MP on NR4A3 transcriptional activity and protein expression. A: dose response of 6-MP effect to augment NR4A3 transcriptional activity. NIH3T3 reporter cells were transfected with NR4A3 fused with luciferase reporter constructs. Cells were seeded in 96-well plates and treated with different doses (0–100 μM) of 6-MP for 24 h. NR4A3 transcriptional activity was measured by quantifying resultant luminescent signals. Luciferase activity (arbitrary units) in DMSO control (0 μM) is set as 1, and the effects of 6-MP are shown as relative fold to this value. Data are means ± SD from 3 separate experiments; n ≥ 3/group. B: 6-MP increases NR4A3 protein expression in L6 myotubes. Fully differentiated L6 myotubes were treated with 0–75 μM of 6-MP for 24 h. Total extracted proteins were then separated by electrophoresis and analyzed by immunoblotting with antibodies against NR4A3. β-Actin level was measured as an internal control. A representative blot is shown. The relative protein signals were quantified densitometrically; data shown are means ± SD (n ≥ 3). *P < 0.05 and **P < 0.01, 6-MP treatments compared with control treatment.
glucose transport 1.4-fold in basal cells ($P < 0.01$), whereas the effect in insulin-stimulated cells was not significant compared with vector control cells (Fig. 3B). In cells overexpressing NR4A3, treatment with 10 μM 6-MP further increased glucose uptake by 60% ($P < 0.01$) in basal cells and by 10% in insulin-stimulated cells ($P = 0.06$) compared with vector cells (Fig. 3B). Thus, an increase in NR4A3 action, whether from overexpression of NR4A3 or by 6-MP activation, upregulated the activity of the glucose transport system in L6 skeletal muscle cells.

**shRNA knockdown of NR4A3 in L6 cells diminishes 6-MP’s effect on glucose transport.** To further determine whether the effect of 6-MP on glucose transport is dependent on NR4A3, we established stable NR4A3 shRNA knockdown L6 cell lines. We collected protein lysates from scramble vector control and NR4A3-knockdown L6 cells treated with or without 6-MP under basal and insulin-stimulated conditions. As shown in Fig. 4A, shRNA knockdown produced a 45% decrease ($P < 0.01$) in NR4A3 expression relative to controls. 6-MP treatment still increased NR4A3 expression in knockdown cells, but levels remained far below those observed in vector control cells. Using these cell lines, we found that the ability of 6-MP to augment basal and insulin-stimulated glucose transport rates was markedly impaired in the NR4A3 knockdown cells when compared with effects observed in the controls (Fig. 4B). Knockdown of NR4A3 alone diminished glucose transport in basal (13% decrease, $P = 0.03$) and insulin-stimulated cells (33% decrease, $P < 0.01$), and transport rates following treatment with 6-MP remained significantly below those in basal and insulin-stimulated control cells (Fig. 4B). These data indicate that the effects of 6-MP on glucose transport are at least partly dependent on NR4A3.

**6-MP treatment augments cell surface GLUT4 in basal and insulin-stimulated L6 myocytes without altering GLUT1 or GLUT4 expression.** To explore the mechanisms by which 6-MP stimulates glucose transport in L6 cells, we first assessed whether GLUT1 or GLUT4, the key glucose transport proteins in skeletal muscle (41), are upregulated by 6-MP. As shown in Fig. 5A, we did not detect any significant changes in GLUT1 or GLUT4 protein levels in L6 cells after treatment with increasing doses of 6-MP. Since recruitment of intracellular GLUT4 to the plasma membrane is the rate-limiting step for insulin-stimulated glucose transport and metabolism in skeletal muscle (15, 41), we next determined whether 6-MP affects translocation of intracellular GLUT4 to the plasma membrane. Using L6 cells transfected with myc-tagged GLUT4 (L6-GLUT4myc), we found that insulin was able to increase cell surface GLUT4 1.6-fold ($P < 0.01$) compared with basal in vehicle-treated control cells and that 6-MP treatment increased cell surface GLUT4 in both basal cells 1.8- to 3.6-fold ($P < 0.01$) and insulin-stimulated cells 2.9- to 4.4-fold ($P < 0.01$) over that in controls, as shown in Fig. 5B. The data lead to the conclusion that 6-MP augments glucose transport through translocation of cellular GLUT4 proteins to the cell surface.

**6-MP stimulated GLUT4 translocation is dependent upon NR4A3.** To examine whether NR4A3 is necessary for 6-MP’s effect on GLUT4 translocation, we first stably overexpressed NR4A3 in L6-GLUT4myc cells and demonstrated an increase in cell surface GLUT4 (Fig. 6A). Specifically, we found that NR4A3 overexpression increased GLUT4 translocation in basal and insulin-stimulated cells compared with vector control cells (both $P = 0.07$) and that 6-MP treatment further augmented surface GLUT4 in insulin-stimulated cells overex-
6-MERCAPTOPURINE ACTIVATES GLUCOSE TRANSPORT VIA NR4A3

Fig. 4. Effects of NR4A3 knockdown and 6-MP on glucose transport in L6 skeletal muscle cells. A: NR4A3 protein levels in NR4A3 knockdown and control L6 cells treated with 6-MP. NR4A3 shRNA stably transfected (NR4A3 knockdown) and control vector-transfected L6 cells were treated with DMSO control (0 μM) or 10 μM 6-MP for 24 h and further incubated in basal or 100 nM insulin for 60 min at 37°C. Total proteins were then separated by electrophoresis and analyzed by immunoblotting with antibodies against NR4A3. β-Actin level was measured as an internal control. A representative blot is shown. The relative protein signals were quantified densitometrically, and data shown are means ± SD (n = 2). Open bars, basal condition; black bars, insulin-stimulated condition. **P < 0.01 compared with basal control vector cells treated with DMSO; &&P < 0.01 compared with insulin-stimulated control vector cells treated with DMSO; ##P < 0.01 compared with insulin-stimulated NR4A3-knockdown cells and with 6-MP-treated control vector cells. B: knockdown of NR4A3 in L6 cells diminished 6-MP’s effect on glucose transport. Vector control and NR4A3 knockdown L6 cells were treated with DMSO (0 μM) or 10 μM 6-MP for 24 h and further incubated in the absence (basal) or presence of insulin (100 nM) for 60 min at 37°C. Measurements of 2-deoxy-D-[3H]glucose uptake were then performed. Data are means ± SD from 3 separate experiments; n = 3/group. Open bars, basal condition; black bars, insulin-stimulated condition. *P < 0.05 and **P < 0.01 compared with basal vector cells treated with DMSO (0 μM); &&P < 0.01 compared with insulin-stimulated vector cells treated with DMSO (0 μM); ##P < 0.01 compared with 6-MP treated NR4A3-knockdown cells compared with 6-MP treated control vector cells under both basal and insulin-stimulated conditions. NS, no significance. 6-MP (10 μM) compared with DMSO (0 μM) within NR4A3 knockdown cells under both basal and insulin-stimulated conditions.

pressing NR4A3 (P < 0.05). Next, we stably suppressed NR4A3 in L6-GLUT4myc cells using shRNA and found that NR4A3 knockdown alone reduced GLUT4 translocation compared with vector control cells (Fig. 6B). Moreover, the ability of 6-MP to increase cell surface GLUT4 was significantly reduced in NR4A3 knockdown cells (P < 0.01) under both basal and insulin-stimulated conditions. These data suggest that 6-MP’s effect to promote GLUT4 translocation is at least in part dependent upon NR4A3.

6-MP increases AS160 phosphorylation in L6 myotubes. We next explored mechanisms of 6-MP’s effect on glucose transport and GLUT4 translocation. Insulin signal transduction is known to increase the phosphorylation of AS160, a Rab-GTPase-activating protein, and phospho-AS160 can further activate Rab proteins that regulate GLUT4 vesicle trafficking and promote GLUT4 translocation (17, 19, 33). Therefore, we studied whether 6-MP can modulate AS160 phosphorylation. We extracted total protein lysates from L6 myotubes treated with 0–75 μM of 6-MP under basal or insulin-stimulated conditions and examined protein expression of phospho-AS160 (Thr642) and total AS160 by Western blot. In Fig. 7, we found that 6-MP increased phospho-AS160 significantly in a dose-responsive manner under both basal and insulin-stimulated conditions. Total AS160 was also increased by 6-MP treatment but not as abundantly as the increase in phospho-AS160, such that 6-MP also increased the ratio of phosphorylated to total AS160. Furthermore, 6-MP did not affect AS160 mRNA levels at doses of 5 and 10 μM, although there was a slight increase observed at doses ≥25 μM, as shown in Fig. 7B. This latter observation is consistent with the predominant effect of 6-MP on AS160 phosphorylation as opposed to an increase in total AS160 per se. These results indicated that induction of AS160 phosphorylation by 6-MP could at least partially contribute to the induction of GLUT4 translocation. Specifically, we studied the dose response of 6-MP stimulation on AS160 (Fig. 7A), NR4A3 protein expression (Fig. 7A), and glucose transport (Fig. 1A) in parallel. We observed that 6-MP produced concomitant increases in glucose transport, NR4A3 expression, and phospho-AS160 with similar dose-responsive characteristics. However, these experiments still did not establish a causal relationship between the increase in NR4A3 and the increase in phospho-AS160.

Effects of 6-MP on protein levels of phosphorylated and total Akt, IRS-1, GSK-3β, and AMPKα and on mRNA level of PGC-1α in L6 cells. In parallel to measuring dose effects of 6-MP on AS160 phosphorylation and NR4A3 expression, we also examined other signaling events that could contribute to stimulation of the glucose transport system. Unexpectedly, we observed that 6-MP decreased basal and insulin-stimulated p-IRS-1 (Tyr612), p-Akt (Thr308), p-GSK-3β (Ser9), and p-AMPKα (Thr172), as shown in Fig. 8A. The diminution of these signaling pathways is consistent with a further downstream effect of 6-MP to stimulate GLUT4 translocation and glucose transport activity and indicates that the increase in AS160 phosphorylation occurs through mechanisms other than Akt. Interestingly, we found that the mRNA level of PGC-1α, which is an important enzyme promoting mitochondrial biogenesis and respiration (40), was slightly but significantly induced by 6-MP (P < 0.05; Fig. 8B).
The ability of 6-MP to modulate phosphorylation of AS160 and Akt occurs independent of NR4A3. Although stimulation of glucose transport (Figs. 3 and 4) and GLUT4 translocation (Fig. 6) by 6-MP are at least partly dependent on NR4A3, we tested whether the effects on AS160 and Akt phosphorylation were similarly NR4A3 dependent. We assessed the impact of NR4A3 overexpression and knockdown on AS160 phosphorylation and the response to 6-MP treatment, as shown in Fig. 9A. We observed that neither NR4A3 overexpression nor knockdown significantly affected the phosphorylated/total AS160 ratio. In addition, 6-MP was still able to increase AS160 phosphorylation in both NR4A3 overexpression and knockdown cells, suggesting that 6-MP’s induction of AS160 phosphorylation was not dependent on NR4A3. Similarly in Fig. 9B, we found that the overexpression of NR4A3 did not affect the reduction in Akt phosphorylation (Thr308) by 6-MP in basal cells or following insulin stimulation, although NR4A3 overexpression alone slightly augmented insulin-stimulated Akt phosphorylation ($P = 0.06$).

**DISCUSSION**

In this study, we have demonstrated for the first time that 6-MP enhances glucose transport in both basal and insulin-stimulated L6 muscle cells, and this effect on glucose transport system activity is the result of increased translocation of GLUT4 glucose transporter to the cell surface. Furthermore, the ability of 6-MP to stimulate glucose uptake is at least in part dependent on NR4A3. First, we found that 6-MP increased both NR4A3 transcriptional activity as well as its level of protein expression, and the 6-MP dose-responsive characteristics for the increase in NR4A3 protein and the increase in glucosetransport and GLUT4 translocation were quite similar. Since we used wild-type L6 cells for glucose transport assay and L6-GLUT4myc cells for GLUT4 translocation assay, the induction levels on L6-GLUT4myc cells were higher due to increased GLUT4 expression. Moreover, 6-MP’s effects on both glucose transport and GLUT4 translocation were enhanced in L6 muscle cells stably overexpressing NR4A3 and diminished following NR4A3 knockdown. These findings provide novel insight regarding the ability of NR4A3 to regulate the glucose transport system in skeletal muscle and add to a growing body of knowledge pertaining to NR4A receptors and metabolism. NR4A family members have been reported to play roles in metabolism in all major insulin target tissues (28, 42). In liver, NR4As can be induced by glucagon and fasting and are correlated with upregulation of gluconeogenesis and protection against hepatic steatosis (42). In adipose tissue, we have reported that NR4A3 enhances the activity of the insulin-responsive glucose transport system and GLUT4 translocation with increased insulin-mediated IRS-1 and Akt phosphorylation (10). In C2C12 skeletal muscle cells, we found that prostaglandin A2 acts as a weak agonist to stimulate glucose transport activity in an NR4A3-dependent manner (43). Also in skeletal muscle, neuromuscular innervation and β-adrenergic activation are found to induce NR4As and result in increased glucose utilization, β-oxidation, and lypolysis (42). The current data are consistent with a role for NR4A receptors in the regulation of glucose uptake and metabolism in muscle. Our current study also shows that the NR4A3 agonist 6-MP increases glucose transport in an NR4A3-dependent manner, and
This represents a novel approach for increasing glucose transport activity using small-molecule transactivators of this orphan receptor.

It is interesting that 6-MP, when used to treat leukemia in children, has resulted in unexplained symptomatic hypoglycemia (7, 12, 44). In this study, we found that 6-MP was able to augment glucose transport and GLUT4 translocation in basal muscle cells even in the absence of insulin. Clearly, hypoglycemia could have resulted from 6-MP activation of NR4A3, causing an increase in skeletal muscle glucose uptake.

To further explore mechanisms underlying 6-MP’s ability to stimulate glucose transport, we assessed its effects on insulin signal transduction. We observed that 6-MP actually diminished phosphorylation of IRS-1, Akt, GSK-3, and AMPK, leading us to the conclusion that 6-MP was acting downstream of these proximal signaling events to promote GLUT4 translocation. These suppressive effects on signal transduction were not explained by 6-MP toxicity since we demonstrated that 6-MP at concentrations (e.g., 5 or 10 μM) used to modulate glucose transport system activity had no significant effect to reduce cell viability. We then quantified expression of PGC-1α since PGC-1α overexpression in mouse muscle caused an increase in insulin-stimulated glucose uptake (1), and PGC-1α mRNA was downregulated by NR4A3 silencing in C2C12 muscle cells (29). Other authors have reported that NR4A3 helped regulate oxidative capacity (27, 29) and that PGC-1α is an important enzyme promoting mitochondrial biogenesis and respiration (1, 40). However, we observed that there was no significant effect of 6-MP on PGC-1α at 6-MP concentrations that clearly enhanced glucose transport activity, although there was a modest increase in PGC-1α mRNA at higher 6-MP concentrations. As a result, we concluded that 6-MP did not appear to modulate the glucose transport system via effects on PGC-1α expression. We were also able to demonstrate that the NR4A3-dependent increase in GLUT4 translocation occurs without the GLUT1 or GLUT4 expression levels being changed in L6 skeletal muscle cells, consistent with our previous findings in 3T3-L1 adipocytes overexpressing NR4A3 (10).

In additional studies to elucidate the mechanisms responsible for 6-MP action, we studied effects on AS160, a Rab-GTPase-activating protein that promotes GLUT4 translocation upon its phosphorylation (17, 19, 33). We observed that 6-MP increased levels of phospho-AS160 and the phosphorylated/total AS160 ratio, which provides a mechanism for the downstream effects of 6-MP on GLUT4 translocation. However, the ability of 6-MP to increase phospho-AS160 was not affected by either NR4A3 overexpression or knockdown. These studies indicated that the 6-MP-mediated increase in phospho-AS160 was independent of NR4A3 in contrast to the effects of 6-MP.

Fig. 6. Effects of 6-MP on GLUT4 translocation in NR4A3-overexpressing and NR4A3 knockdown L6-GLUT4myc cells. (A): GLUT4 translocation in NR4A3-overexpressing L6-GLUT4myc cells. (1): NR4A3 protein levels in stable LacZ control vector-transfected (VEC) and NR4A3-transfected (OE) L6-GLUT4myc cells. β-Actin was measured as an internal control. The representative image is shown. Bands are from the same exposure; irrelevant lanes in between are removed to simplify the figure. (2): LacZ vector control or NR4A3-overexpressing L6-GLUT4myc cells were treated with DMSO control (0 μM) or 10 μM 6-MP for 24 h, followed by incubation in the absence (basal) or presence of 100 nM insulin for 30 min at 37°C. Quantitative analysis of GLUT4 translocation by measurement of cell surface myc epitope (using anti-myc antibody) was performed. Cell surface GLUT4 signal in basal vector control cells (arbitrary units) is set as 1, and the effects of insulin and 6-MP are expressed relative to this value. Data are means ± SD from 3 separate experiments; n = 3/group. Open bars, basal condition; black bars, insulin-stimulated condition. **P < 0.01 compared with basal LacZ control vector cells treated with DMSO; ##P < 0.01 compared with insulin-stimulated LacZ control vector cells treated with DMSO; P = 0.07, overexpression cells compared with vector controls without 6-MP treatment under both basal and insulin-stimulated conditions; &P < 0.05, 6-MP treatment compared with DMSO (0 μM) in insulin-stimulated overexpressing cells. (B): GLUT4 translocation in NR4A3 knockdown L6-GLUT4myc cells. (1): NR4A3 protein levels in stable control VEC and NR4A3 shRNA knockdown (KD) L6-GLUT4myc cells. β-Actin was measured as an internal control. The representative image is shown. (2): vector control or NR4A3 knockdown L6-GLUT4myc cells were treated with DMSO (0 μM) or 10 μM 6-MP for 24 h, followed by incubation in basal or 100 nM insulin state for 30 min at 37°C. Then GLUT4 translocation was measured as described in A. Data shown are means ± SD (n = 3). Open bars, basal condition; black bars, insulin-stimulated condition. *P < 0.05 and **P < 0.01 compared with basal LacZ control vector cells treated with DMSO; #P < 0.05 and ##P < 0.01 compared with insulin-stimulated LacZ control vector cells treated with DMSO; & & P < 0.01. 6-MP-treated NR4A3 knockdown cells compared with 6-MP-treated control vector cells under basal and insulin-stimulated conditions. NS, no significance for comparing 6-MP (10 μM) with DMSO (0 μM) within basal NR4A3 knockdown cells.
on glucose transport activity and GLUT4 translocation that were at least in part dependent on NR4A3. Given that the increase in phospho-AS160 would predictably promote GLUT4 translocation (17, 19, 33), we infer that the ability of 6-MP to augment glucose transport system activity occurs via NR4A3-dependent and NR4A3-independent mechanisms, the latter involving an increase in phospho-AS160. Although global AS160-knockout mice showed impaired glucose uptake in muscle (18), it would be interesting to ascertain the degree to which AS160 knockdown would reduce the ability of 6-MP to stimulate GLUT4 translocation and transport activity.

The data indicate that 6-MP acts downstream to stimulate GLUT4 translocation in the absence of insulin via NR4A3-dependent and -independent mechanisms, the latter involving an increase in phospho-AS160. Although global AS160-knockout mice showed impaired glucose uptake in muscle (18), it would be interesting to ascertain the degree to which AS160 knockdown would reduce the ability of 6-MP to stimulate GLUT4 translocation and transport activity.

The data indicate that 6-MP acts downstream to stimulate GLUT4 translocation in the absence of insulin via NR4A3-dependent and -independent mechanisms while at the same time suppressing proximal steps in insulin signal transduction. This led to higher rates of both basal and insulin-stimulated glucose transport rates with reduced ability of insulin to stimulate glucose transport above basal when compared with control cells. We have previously shown that overexpression of NR4A3 per se resulted in a modest enhancement of signaling through Akt in C2C12 muscle cells and adipocytes (10, 43). Here, NR4A3 overexpression alone in L6 muscle cells slightly increased insulin-stimulated p-Akt with less effect to increase insulin-stimulated glucose transport than to increase basal uptake. Another study demonstrated that NR4A3 transgenic mice displayed improved glucose tolerance without increased insulin sensitivity (27). In any event, the current data combined with our previous results indicate that the NR4A3-independent effects of 6-MP likely explain the suppression of proximal insulin signal transduction as well as the increase in phospho-AS160. In fact, the insulin-independent activation of AS160 is thought to mediate the increased muscle glucose uptake as a result of exercise and muscle contraction (6, 9, 14, 34). Muscle contraction can stimulate glucose transport partially via the activation of AMPK independent of insulin signaling (32, 41); however, AMPK activation did not participate in 6-MP’s induction of glucose uptake since 6-MP also decreased basal and insulin-stimulated phospho-AMPKα (Thr172).

Fig. 7. Effects of 6-MP treatment on protein levels of phosphorylated and total Akt substrate of 160 kDa (AS160) and on mRNA level of AS160 in L6 skeletal muscle cells. A: dose effects of 6-MP on protein levels of phosphorylated and total AS160 compared with NR4A3 protein expression in L6 myotubes. L6 myotubes were treated with DMSO vehicle control (0 μM) or 5–75 μM 6-MP for 24 h and further incubated in basal or 100 nM insulin for 60 min at 37°C. Total proteins were then separated by electrophoresis and analyzed by immunoblotting with antibodies against phosphorylated AS160 (p-AS160 Thr642), total AS160, and NR4A3. β-Actin was measured as an internal control. Representative blots are shown. The relative protein signals were quantified densitometrically, and data shown are means ± SD (n ≥ 3). *P < 0.05 and **P < 0.01 compared with basal control (0 μM); #P < 0.05 and ##P < 0.01 compared with insulin-stimulated control (0 μM). B: dose effects of 6-MP on mRNA level of AS160 in L6 myotubes by RT-PCR. L6 myotubes were treated with DMSO control (0 μM) or 5–75 μM 6-MP for 24 h. Total RNAs were extracted and then RT-PCR and gel electrophoresis were conducted for the analysis of mRNA level of AS160. 18S was measured as an internal control. Representative gel images are shown. Relative mRNAs were quantified densitometrically, and data shown are means ± SD (n ≥ 2).
Several findings in this study warrant further investigation. The NR4A3-dependent mechanisms by which 6-MP increases GLUT4 translocation in skeletal muscle have not been determined. We examined the Akt/AS160 pathway in regulation of GLUT4 translocation in skeletal muscle. In the future, we plan to assess other components that may potentially mediate muscle glucose transport system, such as the aPKC pathway, TC10 pathway, or the even more downstream Rab and Rac proteins, actin remodeling, and the proteins mediating GLUT4 vesicle fusion (e.g., IRAP, V-SNARE, and VAMP2). It is also interesting to examine whether 6-MP changes membrane trafficking dynamics such that GLUT4 exocytosis is favored over endocytosis. In addition, the observed increase in phospho-AS160 together with a diminution in Akt phosphorylation suggest that 6-MP augments phosphorylation of AS160 via an unknown mechanism that does not involve Akt. Future microarray analysis of NR4A3 overexpression or 6-MP-treated cells could be informative in terms of identifying genes and encoded proteins.
that help mediate these effects. Importantly, it would also be of interest to identify other small molecules that transactivate NR4A3 and develop these molecules as insulin-sensitizing, glucose-lowering agents in diabetes. The action of 6-MP to augment muscle glucose uptake via downstream actions without an increase in proximal insulin signaling could be exploited therapeutically. As a purine antimetabolite drug used to treat leukemia, 6-MP at high doses is not an ideal candidate to treat diabetes. However, it may be possible to identify 6-MP structural variants that no longer interfere with purine metabolism but still retain their ability to transactivate NR4A3.

In conclusion, in this study we have demonstrated that 6-MP enhances activity of the glucose transport system via a mechanism that in part requires NR4A3 nuclear receptor even in the absence of insulin. Specific findings include the following: 1) 6-MP increases both NR4A3 expression and NR4A3 transcriptional activity; 2) 6-MP enhances glucose transport in both basal and insulin-stimulated cells through an NR4A3-dependent mechanism; 3) the increase in glucose transport rates by 6-MP is due to an NR4A3-dependent increase in cell surface GLUT4, even in the absence of insulin, without any alteration in the cellular content of GLUT4 or GLUT1 proteins; and 4) 6-MP also increased phospho-AS160 levels, which could contribute to the increase in GLUT4 translocation. However, this latter action of 6-MP was not dependent on NR4A3, indicating that there are NR4A3-dependent and independent actions leading to the increase in glucose transport system activity. These primary findings provide a novel proof of principle that small-molecule NR4A3 agonists can act downstream to increase muscle glucose uptake. Future studies of small-molecule transactivators of NR4A3 will potentially shed light on new treatments for insulin resistance in type 2 diabetes and other metabolic disorders.

ACKNOWLEDGMENTS

We thank Dr. Amira Klip from the University of Toronto for generously providing us with the L6-GLUT4myc cells. We are also grateful for the support of the University of Alabama at Birmingham Diabetes Research and Training Center (P60-DK-079626).

GRANTS

This work was supported by grants from the National Institute of Diabetes and Digestive and Kidney Diseases (DK-083562 and DK-038764 to W. T. Garvey), the Merit Review Program of the Department of Veterans Affairs (W. T. Garvey), and the Alabama Drug Discovery Alliance (W. T. Garvey and Y. Fu).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Q.L. contributed to the conception and design of the research; Q.L., X.Z., L.X., Y.F., and W.G. approved the final version of the manuscript; Q.L., Y.F., and W.G. edited and revised the manuscript; Q.L., X.Z., L.X., Y.F., and W.G. prepared the figures; Q.L. drafted the manuscript; Q.L., Y.F., and W.G. performed the experiments; Q.L. analyzed the data; Q.L. interpreted the results of the experiments; Q.L. prepared the figures; Q.L. performed the experiments; Q.L., Y.F., W.G. edited and revised the manuscript; Q.L., X.Z., L.X., Y.F., and W.G. approved the final version of the manuscript.
REFERENCES


