Human Adenovirus Type 36 Enhances Glucose Uptake in Diabetic and Nondiabetic Human Skeletal Muscle Cells Independent of Insulin Signaling

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OBJECTIVE—Human adenovirus type 36 (Ad-36) increases adiposity but improves insulin sensitivity in experimentally infected animals. We determined the ability of Ad-36 to increase glucose uptake by human primary skeletal muscle (HSKM) cells.

RESEARCH DESIGN AND METHODS—The effect of Ad-36 on glucose uptake and cell signaling was determined in HSKM cells obtained from type 2 diabetic and healthy lean subjects. Ad-2, another human adenovirus, was used as a negative control. Gene expression and proteins of GLUT1 and GLUT4 were measured by real-time PCR and Western blotting. Role of insulin and Ras signaling pathways was determined in Ad-36–infected HSKM cells.

RESULTS—Ad-36 and Ad-2 infections were confirmed by the presence of respective viral mRNA and protein expressions. In a dose-dependent manner, Ad-36 significantly increased glucose uptake in diabetic and nondiabetic HSKM cells. Ad-36 increased gene expression and protein abundance of GLUT1 and GLUT4, GLUT4 translocation to plasma membrane, and phosphatidylinositol 3-kinase (PI 3-kinase) activity in an insulin-independent manner. In fact, Ad-36 decreased insulin receptor substrate-1 (IRS-1) tyrosine phosphorylation and IRS-1– and IRS-2–associated PI 3-kinase activities. On the other hand, Ad-36 increased Ras gene expression and protein abundance, and Ras siRNA abrogated Ad-36–induced PI 3-kinase activation, GLUT4 protein abundance, and glucose uptake. These effects were not observed with Ad-2 infection.

CONCLUSIONS—Ad-36 infection increases glucose uptake in HSKM cells via Ras-activated PI 3-kinase pathway in an insulin-independent manner. These findings may provide impetus to exploit the role of Ad-36 proteins as novel therapeutic targets for improving glucose handling. ***Diabetes* 57:1805–1813, 2008**

Increasing prevalence of type 2 diabetes and insulin resistance is a major health and economic concern (1,2) and necessitates more effective prevention and treatment strategies. Intense search for identifying novel agents that may provide therapeutic targets for better management of diabetes is underway (3,4). Human adenovirus Ad-36 is such a novel candidate that increases adiposity but enhances insulin sensitivity in experimentally infected rats (5), an effect that is robust and reminiscent of the thiozolinediones (6,7). After a single inoculation of Ad-36, fat depot weight increased by >60%, but the fasting insulin levels and homeostasis model assessment (HOMA) index were ~50% lower in rats up to 7 months later (5). Therefore, we postulated that Ad-36 increases glucose uptake in infected tissue, which may contribute in enhancing whole-body insulin sensitivity.

This study investigated the ability of Ad-36 to enhance glucose uptake by skeletal muscle. Skeletal muscle is the largest organ of the human body and is a major site of glucose disposal and insulin action (8). Therefore, exploiting the ability of Ad-36 to enhance glucose uptake by skeletal muscle may provide a novel therapeutic target to treat glycemic disregulation in humans.

In a stepwise approach, we investigated how Ad-36 influences the biomarkers of insulin sensitivity and glucose uptake. First, we determined whether Ad-36 increases glucose uptake in primary skeletal muscle cells from healthy lean and diabetic subjects. Next, the effect of Ad-36 on glucose transporters and their upstream cellular signaling, including phosphatidylinositol 3-kinase (PI 3-kinase) and its activators, was determined. Adenovirus type 2, a human adenovirus that is not adipogenic in animals (9), was used as a negative control. The following experiments showed that Ad-36 activates PI 3-kinase and increases glucose uptake in nondiabetic and diabetic human skeletal muscle (HSKM) cells. Activation of PI 3-kinase by Ad-36 requires Ras signaling but not insulin signaling pathway.

RESEARCH DESIGN AND METHODS

BSA and the protease inhibitors, phenylmethylsulfonyl fluoride, and all other reagent grade chemicals were purchased from Sigma (St. Louis, MO). Skeletal muscle cell growth medium (SkGM) (Cambrex, Walkersville, MD); fetal bovine serum (FBS) (Hyclone, Logan, UT); GLUT1 antibody (Chemicon, Temecula, CA); monoclonal GLUT4 (1F8) antibody (R&D Systems, Minneapolis, MN); polyclonal antibody to insulin receptor β-subunit (IRβ) (Santa Cruz Biotechnology, Santa Cruz, CA); secondary horseradish peroxidase–conjugated antibody, protein A Sepharose, and chemiluminescence reagents (ECL kit) (Amersham, Arlington, IL); nitrocellulose membrane, electrophoresis equipment, Western blotting reagents, and protein assay kit (Bio-Rad, Hercules, CA); anti-p85, phosphotyrosine, insulin receptor substrate-1 (IRS-1), IRS-2, and Ras polyclonal antibodies and pKD-Ras-v1 plasmid or pKD-neg control-v1 plasmid (catalog no. 62-214 or 62-002, respectively; Upstate Biotechnology, Lake Placid, NY); and [^{3}H]2-deoxy-D-glucose and [^{32}P]ATP (NEN Life Science, Boston, MA) were purchased.

Effect of Ad-36 or Ad-2 on in vitro glucose uptake by HSKM cells obtained from diabetic and nondiabetic human volunteers was determined. Next, the effect of the viruses on *GLUT1* and *GLUT4* abundance and membrane

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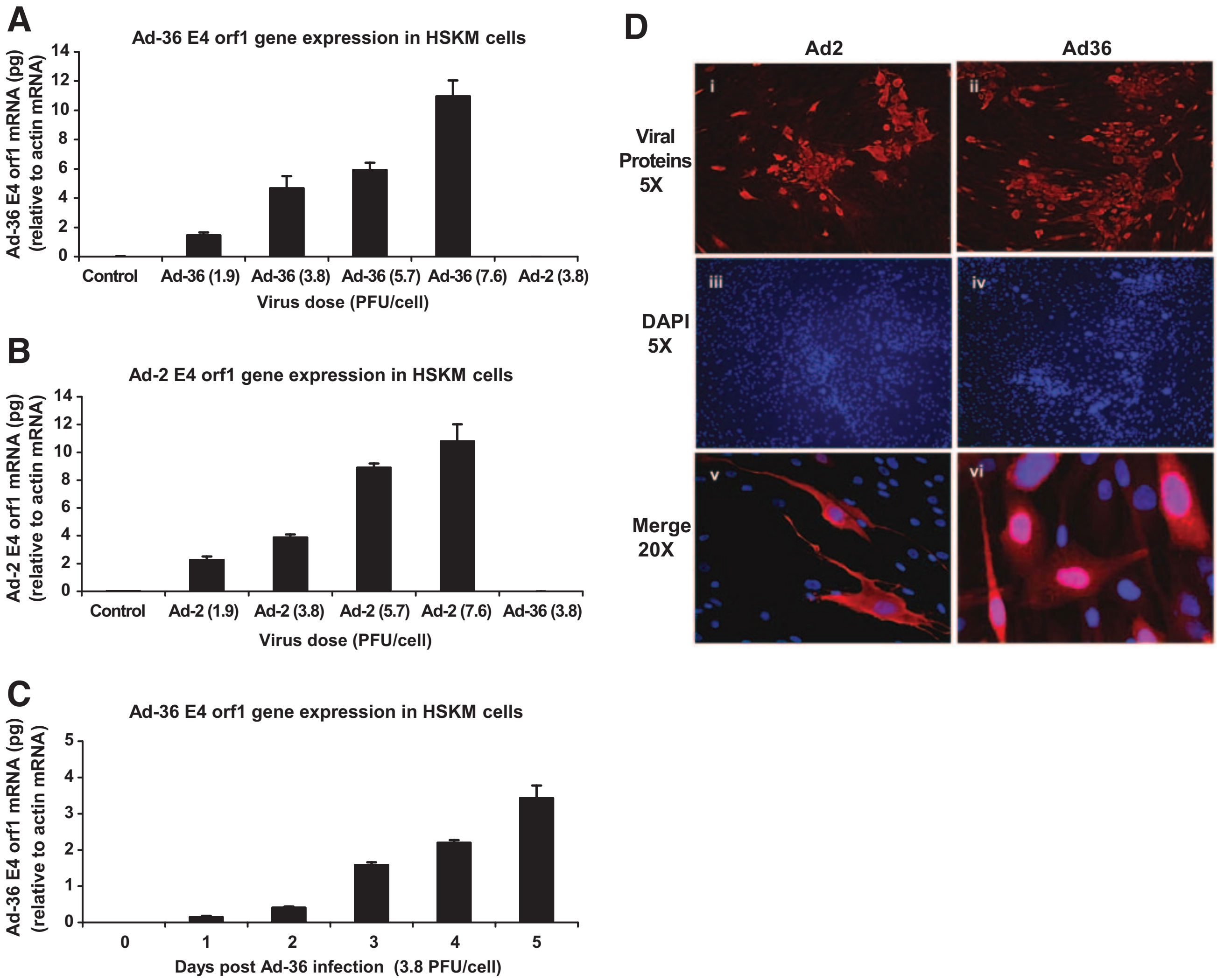


FIG. 1. Expression of Ad-2 and Ad-36 genes and proteins in HSKM cells. *A*: Effect of Ad-36 dose on Ad-36 E4 orf1 gene expression on day 6 after infection. Ad-36 E4 orf1 expression was not detected in control and Ad-2–infected cells. *B*: Effect of Ad-2 dose on Ad-2 E4 orf1 gene expression on day 6 after infection. Ad-2 E4 orf1 expression was not detected in control and Ad-36–infected cells. *C*: The time course of Ad-36 E4 orf1 gene expression in Ad-36–infected cells (3.8 PFU/cell). All E4 orf-1 gene expressions were normalized to β-actin and expressed as nanograms mRNA. HSKM cultures from lean, insulin-sensitive donors were used. Measurements were performed in three experiments, and data are presented as means ± SE. *D*: HSKM cells were infected with Ad-36 or Ad-2 (3.8 PFU/cell) and processed for immunofluorescence 5 days later. Ad-36 and Ad-2 viral particles were identified using anti-Ad36 rabbit polyclonal antibodies and rabbit polyclonal anti–Ad-2 antiserum, respectively. Nuclei were stained with DAPI. *i* and *ii* show cells expressing Ad-2 or Ad-36 proteins, respectively, and *iii* and *iv* show DAPI-stained nuclei. *v* and *vi* show ×20 images of cells expressing Ad2 or Ad36 proteins, respectively.

translocation and PI 3-kinase, Ras, and insulin signaling pathways was determined. Finally, by attenuating Ras with RNAi, we tested the hypothesis that Ad-36 increases glucose uptake in HSKM cells by activating PI 3-kinase via Ras signaling. The individual assays are described in details as follows.

Isolation and culture of HSKM cells. Cells were isolated from the vastus lateralis muscle biopsy and grown as described previously (8). Protocol to obtain muscle biopsies was approved by the human investigations committee of the Pennington Biomedical Research Center. Briefly, muscle biopsies were obtained with a needle from the vastus lateralis muscle from six healthy lean men (age 31.8 ± 4.3 years; BMI 23.2 ± 1.04 kg/m^2; mean ± SE) and six men with type 2 diabetes (age 62.3 ± 2.8 years; BMI 33.5 ± 3.8 kg/m^2; mean ± SE). Approximately 50 mg muscle tissue was minced with surgical scissors and digested by 0.55% trypsin and 2.21 mmol/l EDTA with constant shaking at 37°C. After centrifugation to remove fat and debris, myoblasts were grown in monolayer culture in SkGM with 10% (vol/vol) FBS, 1% (vol/vol) antibiotics (10,000 units/ml penicillin G and 10 mg/ml streptomycin), 2 mmol/l glutamine, and 25 mmol/l HEPES (pH 7.4).

Virus preparation. Ad-2 and Ad-36 viral stocks were prepared by propagation of American Type Culture Collection (ATCC; catalog numbers VR846 and VR913, respectively) viral stocks in ATCC A549 cells (catalog number CCL185) as described previously (10). Viral titers (plaque-forming units [PFU]) were determined by plaque assay (11), and cell inoculations were expressed as PFU/cell.

Infection of HSKM cells with Ad-2 and Ad-36. Cells were maintained in SkGM medium (Cambrex) with 10% FBS. Antibiotic was removed before Ad-36 infection. Unless otherwise indicated in figure legends, 80% confluent HSKM cells were inoculated with Ad-36 or Ad-2 at a dose of 3.8 PFU/cell. After 1 h, viruses were removed, cultures were washed, and complete medium was added. Successful infection was ascertained by determining expressions of respective viral genes (E4 orf-1) by quantitative RT-PCR and by expressions of viral proteins in the appropriate groups.

Immunofluorescence. HSKM cells were grown on chamber slides (Lab-Tek) to 70–80% confluency, serum starved overnight, and infected with Ad-36 or Ad-2 (3.8 PFU/cell). After 5 days, the cells were fixed in 4% paraformaldehyde and processed for immunofluorescence. Ad-36 and Ad-2 viral proteins were identified using anti–Ad-36 rabbit polyclonal antibodies and rabbit polyclonal anti–Ad-2 antiserum, respectively. Secondary antibodies were goat anti-rabbit Alexafluor 594 (Invitrogen). Slides were mounted in ProLong Gold antifade reagent with DAPI (Invitrogen). Images were acquired on a Zeiss Axioplan 2 (Everest) using LD Plan Neofluar ×20 objective and Photometrics Cool Snap HQ camera. All images were processed using Image J software (National Institutes of Health).

Ras siRNA vector transfections. Three days after Ad-36 infection of HSKM cells, Ras siRNA transfections were performed using pKD-Ras-v1 vector or pKD-neg control-v1 vector at 2 and 4 μl/ml FuGENE HD transfection reagent from Roche (Indianapolis, IN). The efficiency >80% of Ras knockdown was confirmed by quantitative RT-PCR.

Quantitative RT-PCR analysis. Confluent HSKM cells were infected with Ad-36, Ad-2, or medium. Total RNA was isolated with TRIzol reagent (Invitrogen) according to the manufacturer's protocol and further purified by RNeasy Mini kit from Qiagen (Valencia, CA). The sequences of the specific primers obtained from IDT (San Diego, CA) were as follows: Ad-36 E4 orf-1, 5′-GGC ATACTAACCCAGTCCGATG-3′ (forward) and 5′-AATCACTCTCTCCAGCAGC AGG-3′ (reverse); Ad-2 E4 orf-1, 5′-CCTAGGCAGGAGGGTTTTTC-3′ (forward) and 5′-ATAGCCCGGGGGAATACATA-3′ (reverse); *Human GLUT1* (SLC2A1, GenBank accession number NM_006516), 5′-GCGGAATTCAATGCTGATGAT-3′ (forward) and 5′-CAGTTTCGAGAAGCCCATGAG-3′ (reverse); and *Human GLUT4* (SLC2A4, GenBank accession number NM_001042), 5′-CGTGGGCGG CATGATT-3′ (forward) and 5′-CCAGCATGGCCCTTTTCC-3′ (reverse).

Gene expressions were determined using ABI PRISM 7700 sequence detector (Applied Biosystems) and a SYBR green detection system (Bio-Rad). A standard was generated using cDNA pooled from experimental samples. Relative expression levels were determined by normalization to β-actin and expressed as arbitrary units.

2-Deoxy-D-glucose uptake. HSKM cells were infected with Ad-36 or Ad-2 as described above. At day 5 after infection, the cells were incubated overnight in serum-free medium. Saline or insulin (100 nmol/l) was added after 14 h of serum starvation during the last 15 min of incubation. Cells were washed twice using PBS, and glucose uptake was determined as described by Klip et al. (12). Briefly, muscle cells were cultured in 24-well plates at 5×10^5 cells/well in SkMG medium containing 5 mmol/l glucose and 2% calf serum for 24 h at 37°C. Culture medium was replaced with serum-free medium and glucose- and pyruvate-free skeletal muscle cell basal medium (no growth factors) containing 10 μCi/ml [^{3}H]2-deoxy-D-glucose 500 μl/well (Perkin Elmer Life Sciences, Boston, MA). The glucose uptake was assessed with or without 10 μmol/l cytochalasin B in KRPH. Plates were incubated for 5 min at room temperature. After washing three times with cold PBS, the cells were lysed with 500 μl/well 0.2 N NaOH for 15 min. A total of 400 μl cell lysate was transferred to scintillation vials, and the radioactivity was determined by scintillation counting. All assays were performed in triplicate in HSKM cells in three separate experiments.

Preparation of muscle plasma membrane and subcellular fractions. GLUT4 protein content in the muscle plasma membrane, low-density microsome (LDM), and high-density microsome (HDM) was determined at day 6 after Ad-36 infection with or without insulin stimulation in HSKM cells from lean, insulin-sensitive donors. Muscle cell plasma and subcellular fractions were obtained as described by Cushman et al. (13). Briefly, on day 6 after inoculation with Ad-36, Ad-2, or medium, muscle cells treated with 0 or 100 nmol/l insulin for 20 min were homogenized in 2.5 volume Lysis buffer (50 HEPES, 150 mmol/l NaCl, 10 mmol/l EDTA, 10 mmol/l $Na_4PH_2O_7$, 100 mmol/l NaF, 2 mmol/l vanadate, 0.5 mmol/l phenylmethylsulfonyl fluoride, and 100 IU/ml trasylol, pH 7.4, containing 250 mmol/l sucrose) by 10 strokes in a PRO 200 homogenizer (PRO Scientific, Oxford, CT). The homogenates were centrifuged at 16,000*g* at 4°C for 20 min. The resulting pellets, consisting of membranes, nuclei, and mitochondria, were resuspended in the same buffer and centrifuged using Beckman ultracentrifuge TL-100 (Beckman, Fullerton, CA) with TLA 100.3 rotor at 100,000*g* for 60 min on a sucrose cushion (38% [wt/wt]) to separate the nuclei/mitochondria from membranes. The plasma membranes between the two layers were collected by centrifugation at 210,000*g* for 60 min. The 16,000*g* supernatants contained cytosolic proteins, HDMs, and LDMs. HDMs and LDMs were collected by ultracentrifugation at 400,000*g* for 45 min. All the centrifugations were performed at 4°C.

Western blot analysis. Western blot assays were conducted as previously described (14). Membranes were incubated with polyclonal or monoclonal antibodies that recognize *IRS-1*, *IRS-2*, *IRβ*, *PI 3-kinase*, protein kinase B (*PKB*)-*p*, *Ras*, *GLUT1*, or *GLUT4* and *β-actin* antibodies. Followed by secondary antibody conjugation with horseradish peroxidase, signals were detected by enhanced chemiluminescence solution. The specific bands were quantitated with scanning densitometry, and the data were normalized to *β-actin* levels.

Total PI 3-kinase and IRS-1– or IRS-2–associated PI 3-kinase activity assays. A total of 500 μg protein was immunoprecipitated with 3 μg PI 3-kinase p85, IRS-1, or IRS-2 polyclonal antibodies to determine PI 3-kinase or IRS-1– or IRS-2–associated PI 3-kinase activity as previously described (15,16). The PI 3-kinase phosphate product was visualized by autoradiography and quantitated by scanning densitometry.

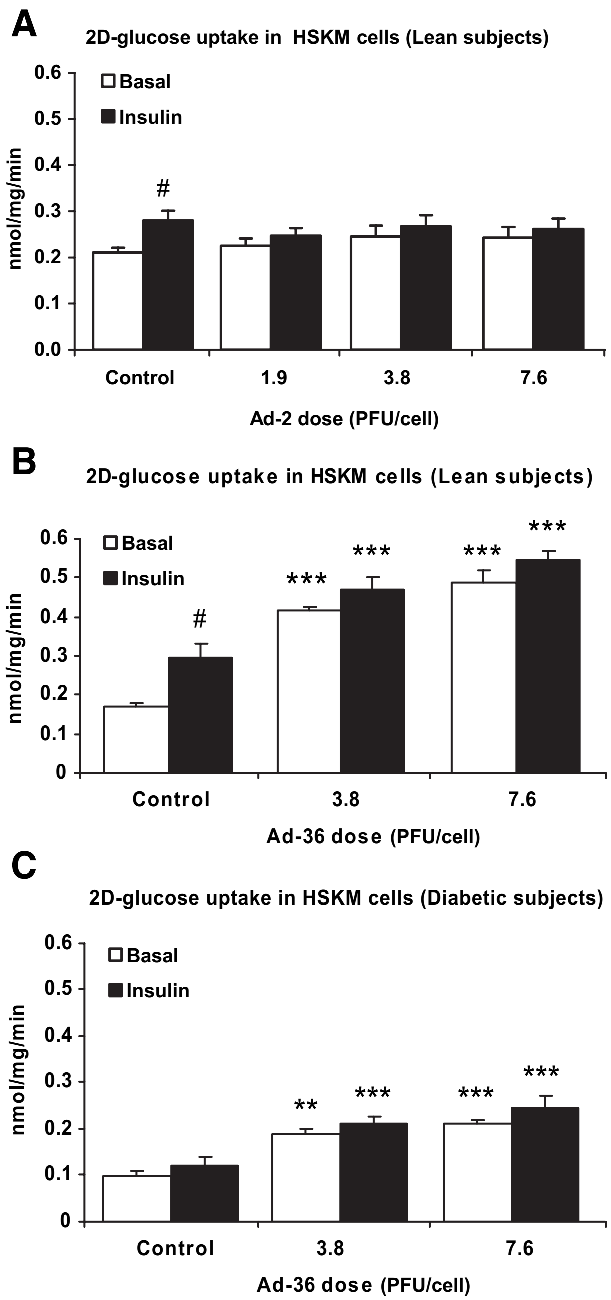
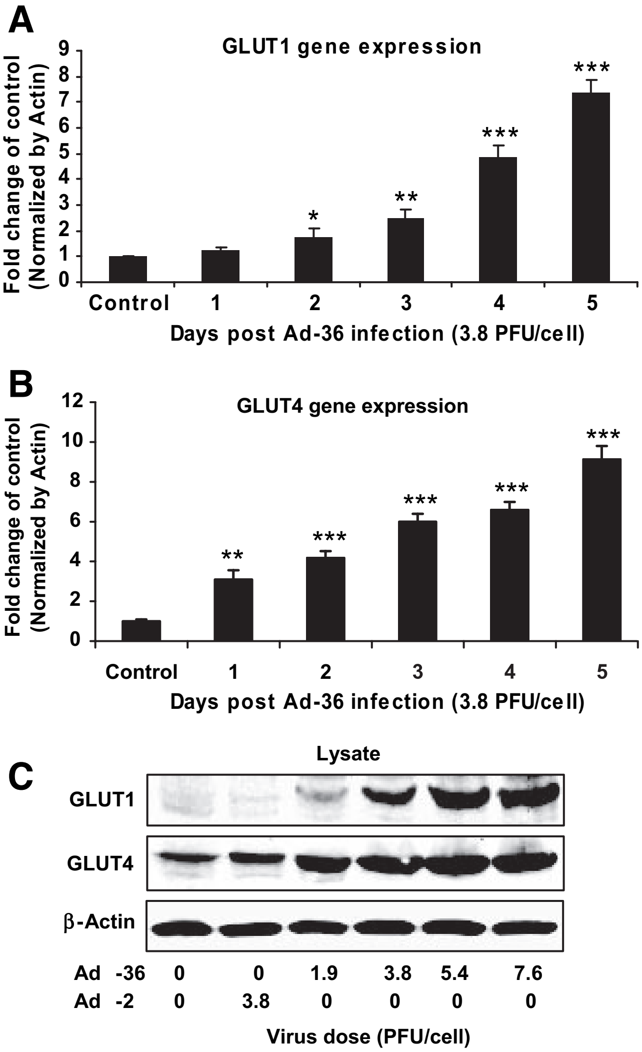


FIG. 2. 2-Deoxy-D-glucose uptake assays in Ad-2– or Ad-36–infected HSKM cells. Human muscle cells were grown in 24-well plates to 80% of confluence in SkGM medium with 10% FBS, 10 units/ml penicillin, and 10 μg/ml streptomycin sulfate. Cells were infected with medium (control) or varying doses of Ad-2 or Ad-36 for 60 min. On day 5 after infection, 2-deoxy-D-glucose (0.2 μCi/ml [1,2-^{3}H]2-deoxy-D-glucose) uptake was determined as described in RESEARCH DESIGN AND METHODS. Means ± SE of three experiments are shown. *A*: Basal or insulin-stimulated glucose uptake in Ad-2–infected HSKM cells from insulin-sensitive, lean healthy donor. #*P* < 0.05, insulin-stimulated vs. basal. *B*: Basal or insulin-stimulated glucose uptake in Ad-36–infected HSKM cells from insulin-sensitive, lean healthy donor in response to Ad-36 infection. #*P* < 0.05, insulin stimulation vs. basal status. ***P* < 0.01 and ****P* < 0.001, Ad-36 vs. respective uninfected controls. *C*: Basal or insulin-stimulated glucose uptake by HSKM cells from insulin-resistant (type 2 diabetic) donor in response to Ad36 infection. ***P* < 0.01 and ****P* < 0.001, Ad-36 vs. respective uninfected controls.



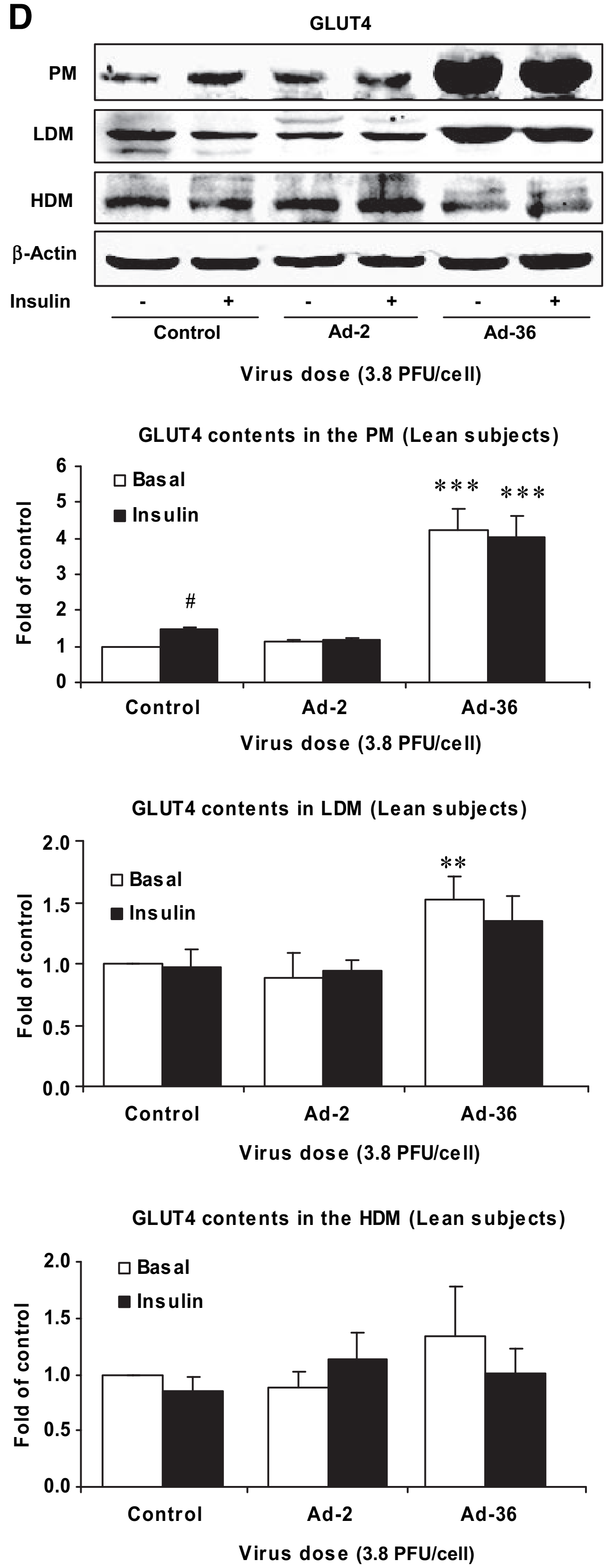


FIG. 3. Effect of Ad-36 on gene expression and proteins of GLUT1 and GLUT4 in HSKM cells. ***A*** **and** ***B*****: Time course studies show increases in GLUT1 and GLUT4 gene expression in response to Ad-36 infection (3.8 PFU/cell). Relative gene expressions were normalized to β-actin levels and expressed in arbitrary units as fold change compared with uninfected controls. Gene expression measurements were performed in three experiments, and data presented as means ± SE. ****P*** **< 0.05, ******P*** **< 0.01, *******P*** **< 0.001, Ad-36–infected vs. uninfected control.** ***C*****: Western blot analysis of GLUT1 and GLUT4 protein abundance in whole lysates of HSKM cells from insulin-sensitive, lean donor at day 6 after infection with Ad-36, Ad-2, or the uninfected control.** ***D*****: Western blot analysis. GLUT4 protein content in the muscle plasma membrane (PM), LDM, and HDM was determined at day 6 after Ad-36 infection with or without 100 nmol/l insulin stimulation in HSKM cells from lean, insulin-sensitive donors as described in RESEARCH DESIGN AND METHODS. The experiments were performed in triplicate. #*****P*** **< 0.05, insulin-stimulated vs. basal condition. ******P*** **< 0.01 and *******P*** **< 0.001, Ad-36 vs. respective uninfected controls.**

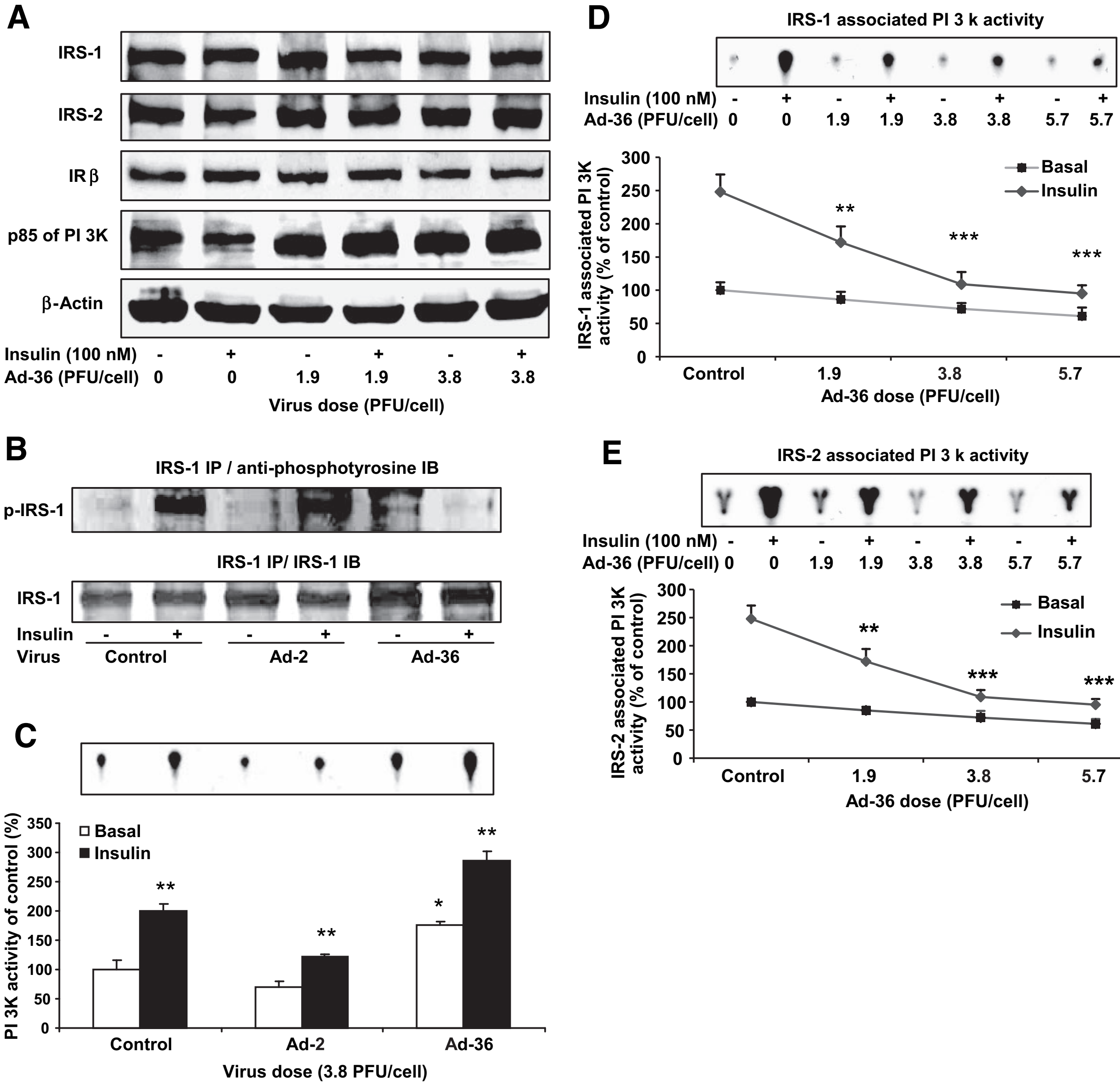


FIG. 4. Increased PI 3-kinase (3K) activity in response to Ad-36 infection is not associated with IRS-1 or IRS-2. HSKM cells from lean subjects were used. Measurements were performed in triplicate experiments, and data are presented as means ± SE. *A*: Western blot analysis for IRS-1, IRS-2, IRβ, and p85 (PI 3-kinase) protein abundance in a virus dose-response study on day 6 after infection. β-Actin was used as a loading control. *B*: Immunoprecipitation (IP) with IRS-1 antibodies followed by Western blot analysis for IRS-1 phosphorylation and IRS-1 on day 6 after Ad-2 or Ad-36 infection (3.8 PFU/cell), with or without insulin stimulation. *C*: Total PI 3-kinase activity was measured at day 6 after Ad-36 or Ad-2 infection with or without insulin stimulation (100 nmol/l) and expressed as percentage of control. *P < 0.05 and **P < 0.01, Ad-36 vs. respective uninfected controls. *D* and *E*: IRS-1– or IRS-2–associated PI 3-kinase activities were measured, respectively, at day 6 after Ad-36 infection at varying doses and with or without insulin stimulation. Data are expressed as percentage of control. **P < 0.01 and ***P < 0.001, Ad-36 vs. respective uninfected controls.

Statistical analysis. The data are presented as means ± SE. Statistical differences in groups were determined by unpaired Student's t test. A P value <0.05 was considered significant.

RESULTS

Ad-36 and Ad-2 infect HSKM cells efficiently. Presence of viral mRNA is evidence of successful viral entry and initiation of viral replication process in a cell. Ad-36 and Ad-2 successfully infected the cells as indicated by the increase in their respective E4 orf-1 gene expressions in a dose- and time-dependent manner (Fig. 1*A*–*C*).

Although the HSKM cells support viral mRNA expression, they do not show cytopathic effect in response to Ad-2 or Ad-36, which makes it difficult to determine relative efficiency of infection of HSKM cells by the two viruses. By using the same viral dose determined as PFU per cell, we observed a fivefold increase in viral mRNA expression between 1.9 and 7.6 PFU/cell for both viruses (Fig. 1*A* and *B*), suggestive of a comparable level of infection by the two viruses. Moreover, Ad36 and Ad2 express viral proteins in HSKM cells and the distribution of the infected cells 5 days after infection appears similar for the two viruses (Fig. 1*D*). Collectively, these results suggest that HSKM cells can be successfully infected with

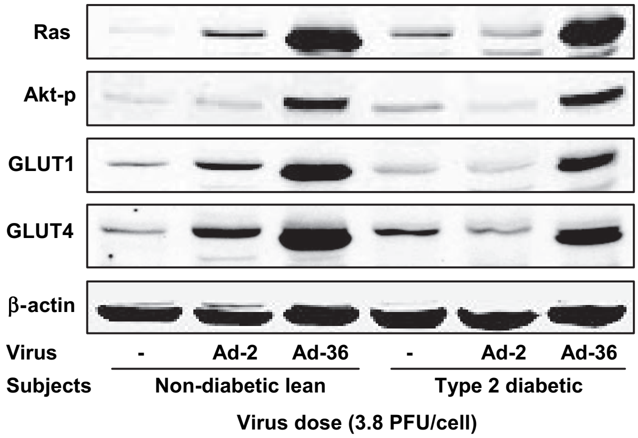


FIG. 5. Ad-36 increases Ras protein abundance, Akt activation, and *GLUT1* and *GLUT4* protein abundance in HSKM cells from lean and diabetic subjects. Western blot analysis of HSKM cells on day 6 after infection with or without Ad-36 or Ad-2. β-Actin was used as a loading control. Western blots are representative of three experiments.

Ad-36 and Ad-2, and the potential to infect these cells is relatively similar for the two viruses.

Ad-36 induces robust increase in glucose uptake in HSKM cells of diabetic and nondiabetic subjects. Ad-36, but not Ad-2, significantly increased [^{3}H]2-deoxy-D-glucose uptake under basal and insulin-stimulated conditions in HSKM cells obtained from nondiabetic subjects (Fig. 2*A* and *B*). Insulin stimulation significantly increased glucose uptake in uninfected control cells, but insulin could not enhance it further in the presence of the viruses. Insulin-stimulated glucose uptake increased 70, 14, and 20% in the control and 3.8 and 7.6 PFU/cell of Ad-36 infection, respectively, compared with their corresponding basal glucose uptakes (Fig. 2*B*). As expected, diabetic muscle cells had much lower glucose uptake compared with that of healthy lean subjects and the basal and insulin-stimulated glucose uptake did not differ significantly. Although HSKM cells from diabetic subjects were less responsive to glucose uptake (Fig. 2*C*), Ad-36 significantly increased 2D-glucose uptake in an insulin-independent manner.

Ad-36 increases *GLUT1* and *GLUT4* gene expression and protein abundance. Glucose uptake in skeletal muscle is dependent on glucose transporter proteins, GLUT1 and GLUT4, of which the latter is regulated by insulin. In HSKM cells, Ad-36 infection significantly increased *GLUT1* and *GLUT4* gene expressions in a time-dependent manner (Fig. 3*A* and *B*) and protein abundance in a dose-responsive manner (Fig. 3*C*), whereas Ad-2 infection did not affect protein levels of the glucose transporters (Fig. 3*C*). Because the translocation of glucose transporters to cell membrane is required for glucose uptake, protein abundance of *GLUT4* in muscle cell membrane fractions was determined (Fig. 3*D*). As expected, insulin increased *GLUT4* abundance in plasma membrane fractions, indicating its translocation to the membrane. *GLUT4* translocation to plasma membrane was also enhanced by Ad-36 but not by Ad-2. However, insulin could not enhance Ad-36–induced membrane translocation of the glucose transporters, suggesting that Ad-36 impaired the ability of insulin to stimulate glucose uptake in the muscle cells (Fig. 3*D*). Ad-2 also appears to block insulin-induced translocation of *GLUT4* by unknown mechanism.

Ad-36–enhanced activation of *PI 3-kinase* is not via the insulin receptor signaling pathway. Western blot analyses for IRS-1, IRS-2, IRβ, and PI 3-kinase in Ad-36–infected muscle cells show that Ad-36 reduced IRS-2 protein abundance at a dose of 3.8 PFU/cell but greatly increased PI 3-kinase protein in a dose-responsive manner (Fig. 4*A*). Ad-36 slightly increased basal IRS-1 tyrosine phosphorylation but dramatically decreased insulin-stimulated IRS-1 tyrosine phosphorylation without affecting IRS-1 protein abundance (Fig. 4*B*), whereas Ad-2 mildly reduced insulin-stimulated IRS-1 phosphorylation in the muscle cells (Fig. 4*B*).

Translocation of *GLUT4* to cell membrane requires PI 3-kinase activation. Because of greater translocation of the glucose transporter in Ad-36–infected HSKM cells, we predicted and observed significantly greater *PI 3-kinase* activation by Ad-36 infection in basal conditions, which was further enhanced by insulin (Fig. 4*C*), whereas Ad-2 infection significantly reduced insulin-stimulated *PI 3-kinase* activation (Fig. 4*C*).

As its key activator, insulin activates *PI 3-kinase* via the insulin receptor signaling pathway involving the interaction of *IRS-1* and *IRS-2* with *PI 3-kinase*. Although insulin enhanced Ad-36–induced *PI 3-kinase* activity, it appears to bypass the signaling pathway in the presence of the virus. In fact, without altering *IRS-1* and *IRβ* protein abundance (Fig. 4*A*), Ad-36 significantly decreased *IRS-1*– or *IRS-2*–associated PI 3-kinase activities in response to insulin (Fig. 4*D* and *E*). Decrease in insulin-stimulated phosphorylation of *IRS-1* in presence of Ad-36 (Fig. 4*B*) further suggests the use of another pathway by insulin to activate PI 3-kinase in the presence of the virus. Next, we investigated the role of Ras in Ad-36–induced PI 3-kinase activation.

Ad-36 infection increased Ras protein abundance in HSKM cells. Ras signaling activates PI 3-kinase (17), which is also a pathway used by human adenovirus type 9 for PI 3-kinase activation (18). Therefore, Ras signaling was a candidate pathway for activation of PI 3-kinase by Ad-36. Ad-36, but not Ad-2, increased Ras, GLUT1, and GLUT4 protein abundance and PKB/Akt phosphorylation in the whole-cell lysates of HSKM cells obtained from nondiabetic and diabetic subjects (Fig. 5).

Ad-36 enhances PI 3-kinase activity and glucose uptake in a Ras-dependent manner. Ad-36 infection increased *Ras* mRNA expression in HSKM cells (Fig. 6*A*). *Ras* siRNA transfection reduced Ras, PI 3-kinase protein abundance, and phospho-Akt levels and attenuated Ad-36–induced increase in *GLUT4* protein abundance in a time-dependent manner but did not affect *GLUT1* abundance (Fig. 6*B*). Attenuation of *Ras* expression also abrogated Ad-36–induced *PI 3-kinase* activation (Fig. 6*C*) and glucose uptake in the muscle cells (Fig. 6*D*). These results show that Ad-36 increases glucose uptake in HSKM cells partially by Ras-mediated PI 3-kinase signaling pathway.

DISCUSSION

In basal condition, Ad-36 infection nearly doubled and tripled glucose uptake in diabetic and lean subjects, respectively (Fig. 2*A* and *B*), and doubled PI 3-kinase activity (Fig. 4*C*). By activating PI 3-kinase via Ras, a known activator of PI 3-kinase (18–20), and by increasing membrane translocation of the glucose transporters, Ad-36 increases glucose uptake in HSKM cells independent of insulin signaling. Ad-36 increased *GLUT1* protein abun-

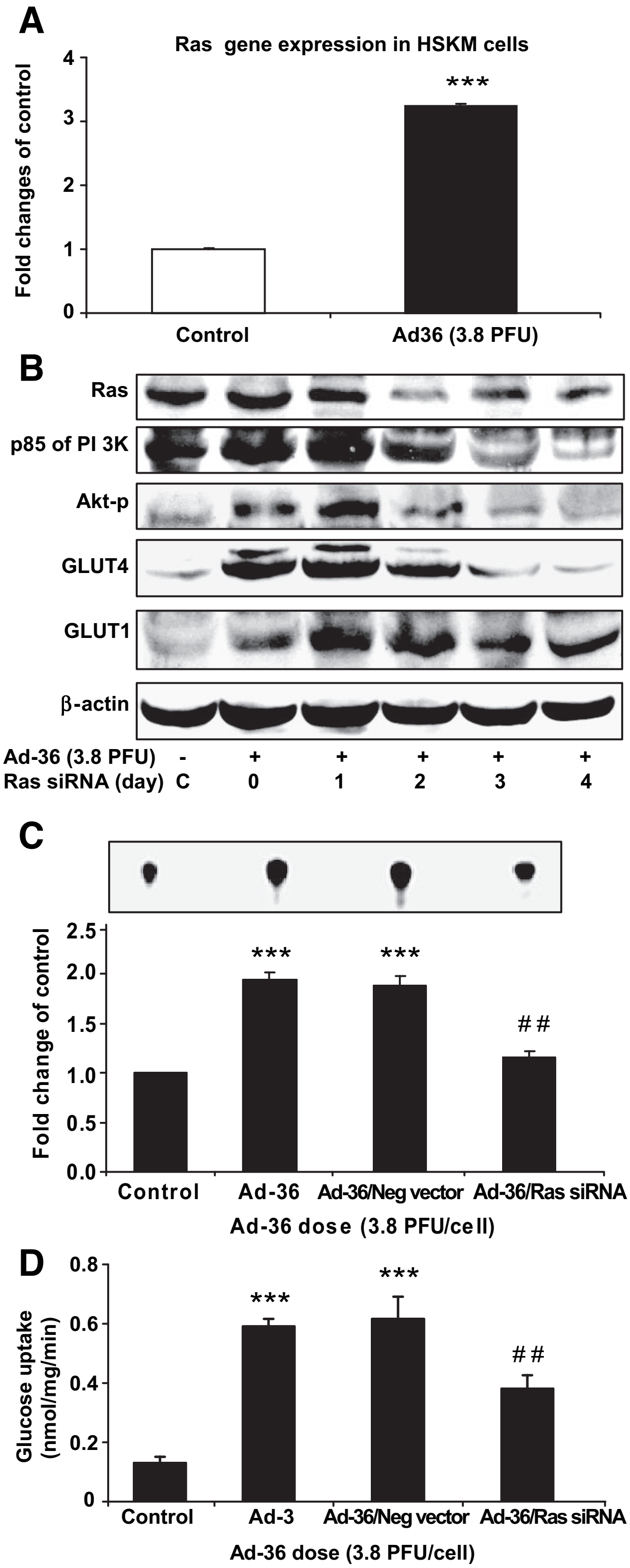


FIG. 6. Ras siRNA transfection reduces PI 3-kinase (3K), Akt-p, GLUT4, and glucose uptake in Ad-36–infected HSKM cells. *A*: Ras gene expression was determined on day 6 after inoculation with Ad-36 (3.8 PFU/cell) or medium (control) in HSKM cells of lean subjects. ****P* < 0.001 Ad-36 vs. control. *B* and *D*: HSKM cultures from lean, insulin-sensitive donors were inoculated with Ad-36 (3.8 PFU/cell) or medium (control). On day 3 after infection, cells were transfected with a 2 μg/ml Ras siRNA and Ras-negative control vectors, respectively. Three days after transfection, assays were conducted in three experiments, and data are presented as means ± SE. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, Ad-36 vs. uninfected controls. ##*P* < 0.01, Ad-36 infection + Ras RNAi vs. Ad-36 infection alone. *B*: Time course of Ad-36 infection in HSKM with or without Ras siRNA transfection. Ras, PI 3-kinase, Akt-p, Glut4, and Glut1 protein abundance were determined by Western blot analysis using β-actin as a loading control. *C*: PI 3-kinase activity was determined on day 6 after Ad-36 infection with or without Ras siRNA or negative control (NC) vectors. Data are expressed as fold change compared with that of the uninfected control. *D*: [^{3}H]2-deoxy-D-glucose uptake was determined 6 days after infection with Ad-36 with or without Ras siRNA transfection.

dance, which may additionally contribute to non–insulin-dependent glucose uptake.

Although the insulin signaling pathway is a well-known activator of PI 3-kinase, Ad-36 induced robust activation of PI 3-kinase without IRS-1 or IRS-2 activation. Ad-36–induced reduction in IRS-1 or IRS-2 activity may be due to inhibitory feedback from increased cellular glucose (21), from the activation of PI 3-kinase itself (22), or from activation of p38 MAPK (23), a kinase commonly induced by adenoviruses (24). In addition, whether Ad-36 directly impairs tyrosine phosphorylation of IRSs remains to be investigated. Even without IRS-1 or IRS-2 activation, insulin did enhance PI 3-kinase activation induced by Ad-36, suggesting the participation of an IRS-independent pathway. It is likely that insulin stimulated tyrosine phosphorylation of insulin receptor, which activated PI 3-kinase via Ras and independent of IRS activation (25). It is unknown why the increased PI 3-kinase activation by insulin in the presence of Ad-36 did not further enhance glucose uptake induced by the virus. Similarly, Ad-2 appears to block insulin-stimulated translocation of GLUT4 translocation to the membrane by an unknown mechanism, which may contribute to a lack of insulin response on glucose uptake in the presence of Ad-2 infection. Thus, Ad-36–induced glucose uptake was independent of insulin but partially depended on Ad-36–enhanced Ras-mediated PI 3-kinase and PKB signaling pathway.

Ad-36 elevated Ras protein abundance in a viral dose-responsive manner, and the effects of Ad-36 on activation of PI 3-kinase, GLUT4 abundance, and glucose uptake were attenuated by inhibition of Ras expression. Ras is an important mediator of anti-apoptotic signals induced by survival factors and promotes survival of many cell systems by activating the PI 3-kinase/PKB pathway (26,27). Role of Ras in increased glucose uptake has been previously reported (28), and other adenoviruses have been reported to activate Ras or PI 3-kinase (18,29,30). However, effect of these adenoviruses on glucose uptake by HSKM cells is unknown.

In summary, Ad-36 infection increased glucose uptake by HSKM cells by increasing *GLUT1* and *GLUT4* gene expression and protein abundance, which appears to be mediated via *Ras*-activated *PI 3-kinase* pathway and independent of insulin signaling (Fig. 7). Considering the major contribution of skeletal muscle to glucose disposal, modulating its ability to uptake glucose may have a major impact on systemic glycemic control. Particularly, the unique and robust ability of Ad-36 to enhance skeletal muscle glucose uptake in an insulin signaling–independent manner is of great interest and may be exploited for developing new drug targets for insulin resistance and type 1 and type 2 diabetes. Future experiments should identify the viral gene(s) responsible and further elucidate the molecular and cellular pathways involved in Ad-36–induced glucose uptake, such as the AMPK pathway, to determine therapeutic targets that mimic the effect.

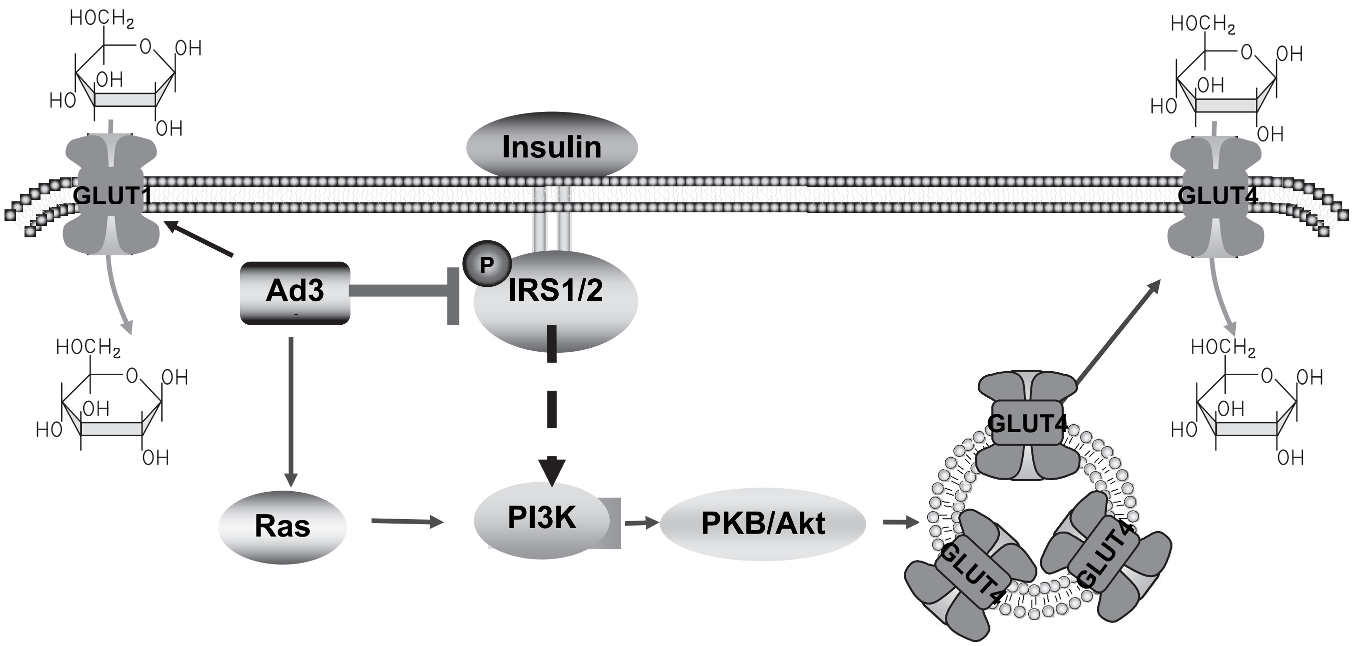


FIG. 7. Proposed mechanism of Ad-36–induced glucose uptake. PI3K, PI 3-kinase.

In conclusion, our findings suggest that the increase of glucose uptake by skeletal muscle may contribute to better glycemic control observed in Ad-36–induced animals (5). Considering the robust effect of the virus on glucose uptake, the cellular and viral protein interaction should be evaluated as therapeutic targets for improving glycemic control in humans.

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