Metabolically Favorable Remodeling of Human Adipose Tissue by Human Adenovirus Type 36

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OBJECTIVE—Experimental infection of rats with human adenovirus type 36 (Ad-36) promotes adipogenesis and improves insulin sensitivity in a manner reminiscent of the pharmacologic effect of thiazolidinediones. To exploit the potential of the viral proteins as a therapeutic target for treating insulin resistance, this study investigated the ability of Ad-36 to induce metabolically favorable changes in human adipose tissue.

RESEARCH DESIGN AND METHODS—We determined whether Ad-36 increases glucose uptake in human adipose tissue explants. Cell-signaling pathways targeted by Ad-36 to increase glucose uptake were determined in the explants and human adipocyte–derived stem cells. Ad-2, a nonadipogenic human adenovirus, was used as a negative control. As a proof of concept, nondiabetic and diabetic subjects were screened for the presence of Ad-36 antibodies to ascertain if natural Ad-36 infection predicted improved glycemic control.

RESULTS—Ad-36 increased glucose uptake by adipose tissue explants obtained from nondiabetic and diabetic subjects. Without insulin stimulation, Ad-36 upregulated expressions of several proadipogenic genes, adiponectin, and fatty acid synthase and reduced the expression of inflammatory cytokine macrophage chemoattractant protein-1 in a phosphotyididylinositol 3-kinase (PI3K)-dependent manner. In turn, the activation of PI3K by Ad-36 was independent of insulin receptor signaling but dependent on Ras signaling recruited by Ad-36. Ad-2 was nonadipogenic and did not increase glucose uptake. Natural Ad-36 infection in nondiabetic and diabetic subjects was associated with significantly lower fasting glucose levels and A1C, respectively.

CONCLUSIONS—Ad-36 proteins may provide novel therapeutic targets that remodel human adipose tissue to a more metabolically favorable profile. Diabetes 57:2321–2331, 2008

Obesity is associated with adverse metabolic profile of adipose tissue, including impaired adipogenesis, lower fatty acid synthase (FAS) and adiponectin, and increased secretion of inflammatory cytokines. Consequently, this contributes to an increase in insulin resistance and a reduction in glucose uptake by the tissue (1–4). Although intentional weight loss could improve insulin resistance and attenuate the adverse metabolic profile, achieving meaningful fat loss and maintaining it long term is very challenging. Instead, a particularly appealing approach proposes to “remodel” the adipose tissue to a more favorable or healthy metabolic profile. For instance, the thiazolidinedione (TZD) class of drugs increases glucose uptake in response to insulin stimulation (5), induces peroxisome proliferator–activated receptor (PPAR)γ2, increases adipogenesis (6), activates phosphotidylinositol 3-kinase (PI3K) (7), reduces the release of inflammatory cytokines (8,9), and upregulates adiponectin secretion (10) and FAS expression (4) in adipose tissue. The metabolically beneficial effects of the TZDs and other remedial candidates, such as benzopyran-derived T33 (11), suggest that adipose tissue remodeling may be a pragmatic approach against the growing epidemic of diabetes. Among the other effects, expansion of adipose tissue by the TZDs appears to offer “storage space” for lipids (5,12) and offset ectopic lipid accumulation in muscle and liver, thereby contributing to insulin sensitivity.

Recently, there is considerable interest in the role of adipose tissue expansion in improving insulin sensitivity. Medina-Gomez et al. (13) showed that PPARγ2 controls adipose tissue expansion and thereby improves insulin sensitivity in ob/ob mice. Kim et al. (14) achieved dramatic improvement in metabolic profile through expansion of adipose tissue in transgenic mice. Despite the massive increase in adiposity, the improved metabolic profile comprised of greater glucose disposal and adiponectin secretion; reduction in serum cholesterol, triglycerides, and inflammation; and induction of expression of PPARγ2 and its target genes (14).

Human adenovirus type 36 (Ad-36) is another novel candidate for improving metabolic profile by expanding adipose tissue. Although Ad-36 increases adiposity (15–17), it enhances insulin sensitivity in experimentally infected rats (18) and reduces serum cholesterol and triglycerides (15–17). Indeed, a single inoculation of Ad-36 increased fat depot weight of rats by >60% but reduced the fasting insulin levels and homeostasis model assessment index by ~50% for up to 7 months later (18), a robust and long-term effect that is reminiscent of TZDs. Moreover, Ad-36 upregulates PPARγ2 expression and induces differentiation and lipogenesis in human and rodent preadipocytes (19–23) and increases glucose uptake in rat adipocytes (22), even in the absence of insulin, which possibly contributes to its insulin-sensitizing effect.

Harnessing certain properties of viruses for beneficial purposes has been creatively used for several years. For instance, even before the advent of antibiotics, the use of bacteriocidal properties of bacteriophage virus has been reported and had a recent resurgence in interest (rev. in

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Infection was confirmed by determining viral gene expression. D-[3H]-deoxy-
tidivity for all experiments. Samples from two or more subjects were used for Ad-36
uptake. Finally, as a proof of concept, nondiabetic and diabetic subjects were screened for the presence of Ad-36
antibodies to ascertain if natural infection with Ad-36 was a significant predictor of improved glycemic control.

**RESEARCH DESIGN AND METHODS**

Human adipose tissue samples were obtained from nine healthy, nondiabetic female patients and one type 2 diabetic female patient undergoing elective liposuction surgeries. The samples were received as material to be discarded and without identifiers. Therefore, approval of the human investigations committee was not required for the study. The donors were Caucasian, aged 28–50 years (mean 39 ± 9.5), and had BMI 18.5–28.2 kg/m² (mean 23.7 ± 3.5). Small chunks of adipose tissue were cultured as explants, as described below, and used to elucidate the effect of Ad-36 infection. Human adipose-derived stem cells (hASCs) were obtained from subjects with BMI 25–30 kg/m², aged 47–58 years. At least three or more technical replicates were used for all experiments. Samples from two or more subjects were used for Ad-36 experiments, except for the diabetic sample. The effect of Ad-2 infection was tested in adipose tissue and hASCs of two separate individuals. An outline of the experiment is presented below, followed by a detailed description of the techniques and assays.

**Effect of Ad-36 on metabolic profile associated with insulin sensitivity.** Adipose tissue explants were infected with Ad-36 or mock infected. Infection was confirmed by determining viral gene expression. D-[3H]-deoxy-glucose was used to determine glucose uptake by the adipose tissue of nondiabetic and diabetic subjects. Adipogenesis was assessed by determining the time course of expression of adipogenic genes (PPARγ2, aP2 [fatty acid–binding protein], lipoprotein lipase [LPL], and glycerol 3-phosphate dehydrogenase) and by counting adipocytes. Considering their important roles in glycemic control, activation of P3K and gene expression and protein abundance of adiponectin and FAS in adipose tissue explants were determined. The gene expression assays conducted for Ad-36 infection were repeated and compared in Ad-2 versus mock-infected adipose tissue explants.

**Requirement of P3K activation for Ad-36–induced alteration in metabolic profile.** Requirement of P3K in Ad-36–induced metabolically favorable remodeling of adipose tissue was determined by using Wortmannin, a specific inhibitor of P3K activity. The effect of Wortmannin on expression of selected cellular genes (PPARγ2, aP2, LPL, platelet endothelial cell adhesion molecule [PECAM]-1), as a marker for angiogenesis induction; macrophage chemoattractant protein [MCP]-1, a marker of proinflammatory cytokine; adiponectin; FAS; and E1orf-1, a viral gene) was determined in adipose tissue explants infected with Ad-36 or mock control subjects. Finally, the effect of Wortmannin on glucose uptake by the explants was determined.

**Role of Ras and insulin receptor signaling in activation of Ad-36–induced P3K activation.** Roles of two major signaling pathways in Ad-36–induced P3K activation were determined. Phosphorylations of insulin receptor (IR), IR substrate (IRS)-1 and -2, and protein abundance of Ras were compared in explants infected with Ad-36 or mock infected. Additional experiments were conducted in hASCs to better elucidate the effect of Ad-36 on signaling pathways. The role of Ad-36 in activation of IRS-1 and P3K was determined with and without stimulation by insulin, a well-known activator of the pathway. Finally, the requirement of Ras, another key activator of P3K, was determined in Ad-36–induced P3K activation and glucose uptake by hASCs. Ras knockdown model, with or without pharmacologic activation and glucose uptake was also determined in Ad-2–infected hASCs.

**Association of natural Ad-36 infection with glycemic control in humans.** Considering the enhanced insulin sensitivity in rats experimentally infected with Ad-36, we hypothesized better glycemic control in humans who are naturally infected with the virus. As a proof of concept, serum samples from nondiabetic and diabetic human subjects enrolled in unrelated studies were screened post hoc and in a blinded manner for the presence of antibodies to Ad-36. A constant-virus-decreasing-serum method was used to determine the presence of Ad-36–neutralizing antibodies as previously described (15,16). Flow neutralization assay is a gold standard and sensitive method for determining neutralizing antibodies. Due to antigenic uniqueness of Ad-36, it is highly specific for detecting Ad-36 antibodies. Available biochemical and anthropometric parameters including glucose and A1C levels were compared between the seropositive and seronegative groups.

**Techniques and assays.** A detailed description of the assays conducted is as follows.

**Ex vivo culture of human adipose tissue explants.** Before infection, explants (~100 mg for gene expression assays, ~20 mg for glucose uptake) were cultured for 3 days in DME/F-12 (no. SH30032.01, Hyclone), 1:1, plus 20% fetal bovine serum (no. SH30070.03; Hyclone) plus antibiotics/antimycotics at 37°C in 5% CO₂, unless otherwise indicated. For P3K inhibition, 0.01 μmol/l Wortmannin (no. W1628, Sigma) was added to cultures postinoculation. Adipose tissue explants differentiated in culture (29) and reduce adipogenic gene expression for a few days in culture. The fall in gene expression can be stabilized by supplementation of the explant media with isobutylmethylxanthine (IBMX) (29). We assessed this in two separate preliminary experiments (online appendix Fig. 1A and B [available at http://dx.doi.org/10.2337/db07-1311]). First, we cultured explants up to 15 days to determine the time course of expression of PPARγ mRNA expression and in the second experiment, we verified that IBMX will stabilize the gene expression of explants. Expression of PPARγ was indeed stabilized by IBMX addition. Therefore, for glucose uptake, adiponectin secretion, and Western blot assays, explant media was supplemented with 0.1 μmol/l IBMX (no. I5879, Sigma) (29). Media was replaced every 2 days. The IBMX supplementation was avoided for the gene expression experiments due to the well-known effect of IBMX on adipogenic gene expression, and the explants were inoculated with virus ~3 days after inoculation.

Isolation and culture of hASCs. hASCs were isolated from liposuction aspirates as previously described (30). Briefly, the stromal-vascular fraction was resuspended in DME/F-12, 1:1, plus 10% fetal bovine serum plus antibiotics/antimycotics and plated at a density of 0.156 ml of tissue digest/cm² as passage 0 (p0) and used for experiments within passages 2–4 (p2–p4). No Ad-36 DNA was detected in these tissues before experimentation.

**Viruses preparation.** Ad-36 was obtained from the American Type Culture Collection (catalog no. VR913) and the plaque purified and propagated in A549 cells (human lung cancer cell line) as described and used previously (15,16). Ad-2 was also obtained from the American Type Culture Collection (catalog no. VR946) and propagated in A549 cells. Viral titers were determined by plaque assay (16) and cell inoculations expressed as plaque-forming units per cell (pfu/cell).

**Ad-36 or Ad-2 infection of human adipose tissue explants.** Explants were infected with 1 h for 100 μl/cm² DME/F-12, 1:1 (mock), or 100 μl/cm² DME/F-12, 1:1, plus Ad-36 or Ad-2 (~10⁹ infectious particles). Following the infection, media and virus were removed and replaced with DME/F-12, 1:1, plus 10% fetal bovine serum plus antibiotics/antimycotics.

**hASCs.** hASCs were seeded at 15,000 cells/cm². In Ad-2 versus mock-infected adipose tissue explants.

**Primers for qRT-PCR.** Gene expression was 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 cyclophilin and expressed as arbitrary units.

**Primers for qRT-PCR.** Primers for human PECAM-1 were purchased from Applied Biosystems. All other primer sequences were as follows. Ad-30E1orf-1 forward: 5’-GGTTAGGGCCAGACAGAG-3’; Ad-30E1orf-1 reverse: 5’-GATACACTGTCTGCACGGAGC-3’; human PPARγ forward: 5’-GGAGATGGCACAGGAGGAAA-3’; human cyclophilin B reverse: 5’-GCAAAACATATCACAA; human PPARγ reverse: 5’-CCAGGCGGCTGTCATGTTCAC-3’; human G3PDH forward: 5’-CTATACAGCATCCTCCAGCACAA-3’; human G3PDH reverse: 5’-cctaggcaggagggtttttc-3’. Relative expression levels were determined by normalization to cDNA and expressed as arbitrary units.
TATGCTTCTCGTGCAGCTT-3'; human FAS reverse: 5'-GCTGCAA CACGCTCTCTCAGT-3'; human MCP-1 forward: 5'-CACCGAGTATCA TATCAAGC-3'; and human MCP-1 reverse: 5'-TGTTTUTACCATGAAATCCTGAA-3'.

**Western blot analysis.** Protein concentrations were quantitated by bicinchoninic acid assay and loaded to the 4–20% or 10% polyacrylamide gel in equal amounts. Proteins then were transferred to a polyvinylidene fluoride membrane. Membranes were blocked in PBS Tween-20 containing 3% BSA and incubated with antibodies in appropriate dilutions. Signals were quantitated by GelPro 3.1 Analyzer software. Equal loading was assessed by normalization to α-β-tubulin or actin abundance. Phosphoprotein abundance was normalized to signal of total protein of interest.

**Antibodies for Western blot analysis.** Antibodies to Ser473 protein kinase B (PKB), total PKB, Ras, secondary antibodies, and α-β-tubulin were purchased (Santa Cruz, CA). Antibodies to adiponectin and FAS were purchased from Abcam (Cambridge, MA). Antibodies to IR-8 were purchased from Upstate (Chicago, IL). Antibodies to IRS-1 and -2 were purchased from Santa Cruz (Santa Cruz, CA).

**Adipocyte counts.** Adipose tissue explants were placed in histological cassettes and fixed overnight in Bouin's solution. After fixation, explants were washed with water for 2 h and placed in 70% ethanol overnight before processing. Tissues were embedded in paraffin, and 6-μm slices were subjected to hematoxylin and eosin staining protocol. Adipocytes were counted using Metamorph software and adipocyte numbers expressed as cells per unit surface area. Six slices each from three individual explants were used for calculation.

**Adiponectin secretion.** Adiponectin was measured in the media of human adipose tissue explants cultured for 3 days postinfection by Western blotting as described above. Adiponectin levels in the media were normalized to total protein of adipocytes expressed as arbitrary units.

**Glucose uptake assays.** [3H]-deoxy-glucose was purchased from Amer sham (Piscataway, NJ). Glucose uptake experiments were performed as previously described (31). Briefly, human adipose tissue explants (~20 mg) were incubated at 37°C, 5% CO2 for 30 min in 500 μl DMEM/12, 1:1, plus 2% BSA. In a pilot experiment, we determined the glucose uptake by adipose tissue explants to be linear up to 40 min of incubation (online appendix Fig. 2). For adequate tissue permeability, a 30-min incubation was used. Explants were washed and added to Krebs-Ringer HEPES buffer plus 1% BSA. Explants were incubated for 15 min in Krebs-Ringer HEPES buffer plus 1% BSA before the addition of 2 μmol/l [3H]-deoxy-glucose and 50 μmol/l glucose to each well and further incubation for 30 min. Explants were washed three times in ice-cold Krebs-Ringer HEPES plus 1% BSA to stop the reaction and to remove unincorporated label. Explants were blotted and incubated for 30 min at 65°C in 1 N NaOH before protein quantitation, and 3H radioactivity was determined by scintillation counting. Data are expressed as picomoles 2-deoxyglucose per milligram protein.

**Ras short hairpin RNA assay.** hASC cells were cultured to 80% confluency in growth media in 100-mm dishes and infected with Ad-36 (3.8 pfu/cell). Four days postinfection, Ras short hairpin RNA (shRNA) vector transfection was conducted using 20 μg pKD-Ras-v1 plasmid (catalog no. 62-214) or pKD-neg control-v1 plasmid (catalog no. 62-002) from Upstate Biotechnology (Lake Placid, NY). Times in Krebs-Ringer HEPES buffer plus 1% BSA. Explants were incubated for 15 min in Krebs-Ringer HEPES buffer plus 1% BSA before the addition of 2 μmol/l [3H]-deoxy-glucose and 50 μmol/l glucose to each well and further incubation for 30 min. Explants were washed three times in ice-cold Krebs-Ringer HEPES plus 1% BSA to stop the reaction and to remove unincorporated label. Explants were blotted and incubated for 30 min at 65°C in 1 N NaOH before protein quantitation, and 3H radioactivity was determined by scintillation counting. Data are expressed as picomoles 2-deoxyglucose per milligram protein.

**RESULTS**

**Effect of Ad-36 on viral gene expression, PKB activation, and glucose uptake by human adipose tissue explants.** Experiments were performed as described in **RESEARCH DESIGN AND METHODS**.

**FIG. 1.** Effect of Ad-36 on viral gene expression, PKB activation, and glucose uptake by human adipose tissue explants. Experiments were performed as described in **RESEARCH DESIGN AND METHODS**. A: Time course E4orf1-1 gene expression after Ad-36 infection normalized to cyclophilin B. B: Representative Western blot analysis of PKB phosphorylation at ser473. Upper panel shows the autoradiograph. Lower panel shows the densitometry means ± SE (n = 3 per group). □, mock; ■, Ad-36. Values normalized to total PKB. *P < 0.01. C and D: Glucose uptake assay in adipose tissue from insulin-sensitive and insulin-resistant donors. Donors were matched for BMI. Representative experiments shown as means ± SE. □, mock; ■, Ad-36. D: Glucose uptake assay in adipose tissue substrate to linoleic (type 2 diabetic) donor 5 days after Ad-36 infection. □, mock; ■, Ad-36. *P = 0.036; **P = 0.035.

**Adipose tissue infection.** Adipose tissue explants were infected with Ad-36, and 51 days postinfection, Ras short hairpin RNA vector transfection was performed (as evident by cells expressing viral proteins) by Ad-36 and Ad2 were subjected to standard hematoxylin and eosin staining protocol. Tissues were embedded in paraffin, and 6-μm sections were subjected to immunoperoxidase staining with antibodies to PPARγ2 and late (LPL, aP2, and glycerol 3-phosphate dehydrogenase) genes of adipogenic cascade, which was visualized by autoradiography and quantitated by scanning densitometry with Quantity 1 1D software version 4.2.1 using Bio-Rad gel documentation. PI3K activity was determined. In a separate experiment, hASCs were infected with Ad-2, Ad-36 (10 pfu/cell), or mock infected and transfected with either pKD-Ras-v1 plasmid or pKD-neg control-v1 plasmid. About 4 days postinfection, glucose uptake was determined and the samples were harvested for Western blot analysis.

**P13K activity assay.** A total of 500 μg of hASCs total protein extract was subjected to immunoprecipitation with 3 μg of P13K p85 polyclonal antiserum (Upstate) to determine P13K activity as previously described (19). The P13K-phospholipid product was visualized by autoradiography and quantitated by scanning densitometry with Quantity 1 1D software version 4.2.1 using Bio-Rad gel documentation.

**Infectivity of hASCs.** Near-confluent hASCs were infected with serial 10-fold dilutions of 100 μl stock of viruses Ad-36 and Ad-2 (triplicate for each dilution) and overlaid with agar. Starting plaque-forming units (calculated using A5340 cells) were 6.5 × 103 per ml and 8 × 103 per ml for Ad-36 and Ad-2, respectively. Cells were fixed 8 days postinfection and stained with 4',6-diamidino-2-phenylindole dihydrochloride (catalog no. D1260; Invitrogen) and adenovirus hexon antibodies (rabbit antiserum catalog no. VR1079; American Type Culture Collection) followed by Alexa-fluor 594 goat anti-rabbit antibodies (catalog no. A-11012; Invitrogen). The number of plaques formed (as evident by cells expressing viral proteins) by Ad-36 and Ad2 were compared for dilutions that used similar plaque-forming units to infect (8 × 104 pfu for Ad-36 and 6.5 × 104 pfu for Ad-36).

**Statistics.** Assays were performed in triplicate and reported as means ± SE. A one-sided Student's t test was used to determine significance (*P < 0.05). Effect of Ras shRNA on glucose uptake was determined in mock, Ad-36−, or Ad2-infected hASCs by two-way ANOVA followed by the Tukey-Kramer test. Analyses were conducted using functions available in the base package of R (32) or SAB.
adenovirus, which is nonadipogenic in vivo and in vitro (23,33), was used as a negative control. In addition, we conducted a plaque-forming unit assay in hASCs. The number of plaques formed in hASCs by nearly equal number of particles of the two viruses were not significantly different ([means ± SD] Ad-36 vs Ad-2 13.7 ± 0.6 vs. 8.7 ± 3.2; \( P = 0.12 \)). Collectively, this indicated that the difference in Ad-36 and Ad-2 in glucose uptake and other adipogenic effects is not due to their differential ability to infect human adipose tissue or hASCs. Despite successful infection of the explants as evident from viral mRNA expression (Fig. 4A), unlike Ad-36, Ad-2 did not induce expression of the above-described genes of adipogenic cascade (Fig. 4B–G). Therefore, subsequently, we focused only on the effect of Ad-36.

**PI3K is required for Ad-36–induced improvement in metabolic profile.** PI3K is a key molecular for several cellular pathways, which is evident from the fact that its inhibition by Wortmannin reduced the expressions of aP2, PPAR-\( \gamma \), MCP-1, and FAS (Fig. 5A). Also, Ad-36 E4orf-1 gene expression requires PI3K activation (Fig. 5B). Furthermore, Ad-36–induced expression of PPAR-\( \gamma \), aP2, LPL, and PECAM-1 in the adipose tissue was dependent on PI3K activation (Fig. 5C–F). Via PI3K activation, Ad-36 suppressed expression of MCP-1, a proinflammatory marker. Wortmannin reversed Ad-36–induced MCP-1 suppression, which also showed that its effect on

**Fig. 3.** Increased adiponectin and FAS in Ad-36 infection of human adipose tissue. Experiments were performed as described in research design and methods. A and B: Time course gene expression after Ad-36 infection as measured by qRT-PCR and normalized to cyclophilin-B. Data are means ± SE. Representative experiment \( n = 3 \) explants per group. \( \square \), mock; \( \square \), Ad-36. C and D: Western blot analysis for adiponectin (media) and FAS protein levels. \( \square \), mock; \( \square \), Ad-36. C: Upper panel: Autoradiograph of Western blot. Lower panel: Densitometry means ± SE \( n = 3 \) per group. Adiponectin protein levels were normalized to total protein content of individual explants. D: Upper panel shows autoradiograph. Lower panel: Densitometry means ± SE \( n = 3 \) per group. FAS protein levels were normalized to \( \beta \)-tubulin. * \( P < 0.05; \) ** \( P < 0.01 \).

**Fig. 2.** Increased adipogenic gene expression and adipocyte cell numbers in Ad-36–infected human adipose tissue explants. Experiments were performed as described in research design and methods. All experiments were representative \( n = 3 \) per group. Data are means ± SE. A–D: Time course gene expressions measured by qRT-PCR and normalized to cyclophilin. \( \square \), mock; \( \square \), Ad-36. E and F: Number of cells per field; \( n = 3 \) (explants) per group. Six slices per explant were used for calculations. \( \square \), mock; \( \square \), Ad-36. \( \times P < 0.05 \). G: Representative sections of adipose tissue used for calculation of the number of cells in \( E \) and \( F \). Sections from Ad-36–infected explants show a greater number of smaller cells on day 21 postinoculation. Red dots indicate cells counted as adipocytes.

FAS and adiponectin, and increases glucose disposal, indicating improved metabolic profile of the tissue.

The effect of Ad-36 on adipose tissue cannot be attributed simply to any viral infection. Ad-2, another human
cellular gene expression is not universally inhibitory in the presence of the virus. Importantly, Ad-36 required PI3K to enhance glucose uptake (Fig. 5G). Collectively, these results demonstrated that Ad-36 induces metabolic changes in adipose tissue via activation of PI3K.

**Ras, but not insulin receptor signaling, is required for Ad-36–induced PI3K activation and glucose uptake.** The effect of Ad-36 on insulin receptor signaling and Ras signaling, the two key activators of PI3K, was determined in adipose tissue explants and in hASCs. Ad-36 reduced the abundance and activation of IR, IRS-1, and IRS-2 in explants but greatly increased Ras protein abundance in explants (Fig. 6A), suggesting that the virus uses Ras signaling rather than insulin receptor signaling for PI3K activation. In hASCs, under basal or insulin-stimulated conditions, Ad-36 activated PKB phosphorylation but blocked IRS-1 tyrosine phosphorylation (Fig. 6B–E), confirming its lack of contribution in Ad-36–induced PI3K signaling. Therefore, we tested the participation of Ras signaling in Ad-36–induced activation of the PI3K pathway. As hypothesized, Ras-specific shRNA abrogated Ad-36–induced PI3K activity (Fig. 7A), indicating Ras as an upstream regulator of Ad-36–induced PI3K activity. Furthermore, as predicted from its role in Ad-36–induced PI3K activation, knockdown of Ras expression by shRNA showed that Ad-36–induced glucose uptake by hASCs is Ras dependent (Fig. 7B). Interestingly, Ad-36 induced a fourfold increase in glucose uptake by hASCs, which could not further be enhanced by insulin stimulation (Fig. 7B), which further demonstrated the robust and insulin-independent effect of Ad-36 on glucose uptake.

Finally, we compared the effects of Ad-36 and Ad-2 on glucose uptake in hASCs. Compared with the mock-infected hASCs, Ad-36, but not Ad-2, increased glucose uptake by ~2.5-fold (Fig. 7C), and Ras abundance knockdown was up to 50% (Fig. 7D). As expected, the Ad-36 group showed greater PI3K pathway activation, as indicated by increased Ser-473 and Thr-308 phosphorylation of Akt/PKB. Ras knockdown reduced Ad-36 protein abundance and Akt/PKB phosphorylation (Fig. 7D), and Ad-2
did not increase Ras abundance, PKB activation, or glucose uptake. Ad-2 protein abundance was not affected by Ras knockdown.

**Natural infection of Ad-36 is associated with better glycemic control in humans.** Blinded post hoc screening for neutralizing antibodies to Ad-36 in nondiabetic as well as diabetic subjects enrolled in unrelated studies showed ~12% prevalence of Ad-36 infection (Table 1). Study 1 included 3 men and 34 women who were nondiabetic or diabetic (31 Caucasians and 6 African Americans) (Table 1A). Study 2 included 16 men and 32 female diabetic subjects (30 Caucasians and 18 African Americans) (Table 1B). Seropositive and seronegative individuals were not significantly different with respect to age, BMI, and blood pressure in respective studies. However, seropositivity was a significant predictor of lower fasting glucose (Table 1A; online appendix Fig. 3) or A1C levels (Table 1B; online appendix Fig. 4). Moreover, within the diabetic group, Ad-36 seropositivity was significantly associated with lower serum cholesterol and LDL cholesterol levels (Table 1B). Although not causational, these findings are remarkably similar to the virus-induced phenotypic patterns.
observed in experimentally infected animals (18) and suggest that natural Ad-36 infection may be an important moderator of glucose disposal in humans.

**DISCUSSION**

Adipose tissue is comprised various cell types, including those of adipogenic, endothelial, and immune lineage. Therefore, adipose tissue explants were mainly used for better representation of the collective response of the constituent cell types (31,34,35). The use of explants allowed for the testing of the potential of Ad-36 to favorably alter the metabolic profile of the entire tissue, including the proadipogenic, angiogenic, and anti-inflammatory effects. A limitation of explant use is their limited utility to study cell signaling by methods such as RNA knockdown. Therefore, hASCs were used to further elucidate molecular mechanism.

Overall, Ad-36 improves the metabolic profile of human adipose tissue, as indicated by greater glucose uptake; reduced expression of MCP-1, a proinflammatory marker; increased FAS and adiponectin levels; and increased expression of adipogenic genes such as PPARγ2, which are associated with better glucose disposal (6,36). This effect of the virus very closely resembles a transgenic mouse model, which shows improved metabolic profile due to adipose tissue expansion through adiponectin overexpression (14). Ad-36–induced glucose uptake is particularly robust, even in basal conditions, which could not be enhanced further by insulin stimulation (Fig. 7B), indicating maximal stimulation of the glucose uptake process by the virus. It is particularly noteworthy that Ad-36 increased the glucose uptake by adipose tissue of even a diabetic subject, suggesting its insulin-independent effect. We reported increased and insulin-independent glucose uptake in human primary skeletal muscle cells infected with Ad-36 (37). Ability of Ad-36 to enhance glucose uptake by adipose tissue and skeletal muscle may collectively contribute to enhanced glucose disposal observed in experimental (18) and natural (Table 1) Ad-36 infection. The cellular targets of Ad-36 action need to be identified to harness this potential of the viral proteins for therapeutic use.

Ad-36 influences adipose tissue metabolic profile in a PI3K-dependent manner. The activation of the PI3K pathway induces cell proliferation and adipogenesis (38,39), increases adiponectin expression (40), reduces inflammatory response (41), and enhances glucose uptake (42) in adipocytes and participates in a number of other cell functions. The pivotal role of PI3K in cellular metabolism was also evident from the reduction in cellular gene expression in the presence of Wortmannin (Fig. 5A and B). PI3K activation is also required for cellular entry by some adenoviruses (43,44), but not all (45), and human adenoviruses such as Ad-5, Ad-9, and Ad-19 are known to activate PI3K (45–48). Since the adipose tissue was infected with Ad-36 before Wortmannin treatment, the effect...
of PI3K inhibition on cellular entry of Ad-36 was not relevant.

Insulin is a well-known activator of PI3K activity. The binding of insulin to the insulin receptor activates insulin receptor's internal tyrosine kinase activity. The activated tyrosine-phosphorylated insulin receptor phosphorylates IRS, which in turn activates PI3K, eventually leading to Glut4 translocation and glucose uptake. While IRS-1 is particularly important for insulin-stimulated PI3K activity and Glut4 translocation in adipose cells (49), Ras signaling also plays a prominent role in activation of PI3K (50,51) and glucose uptake (52). Therefore, the roles of insulin and Ras signaling were determined in Ad-36–induced PI3K activation.

Ad-36 blocked IRS-1 activation by insulin in hASCs, perhaps due to negative feedback from activated PI3K, as shown by Tanti et al. (53) and reviewed by Ye (54). Therefore, enhanced activation of PI3K signaling by insulin in the presence of the virus is unlikely to be IRS-1 mediated. Instead, it is probably mediated via the Ras signaling pathway, as shown by Sakaue et al. (55), which showed that tyrosine phosphorylation of IR by insulin activates Ras via son of sevenless (SOS), a guanine nucleotide exchange protein, and such activation is independent of IRS-1 activation.

Ad-36–induced glucose uptake as well as the viral gene expression appears to be dependent on PI3K and Ras, which are also recruited by various other viruses for their replication (43,56–58). It is unclear if Ad-36 requires Ras or PI3K for directly inducing the downstream glucose uptake or indirectly for viral gene expression itself. Our data suggest that either or both possibilities may exist. Ad-36 may upregulate the Ras-PI3K pathway to increase viral gene expression, which in turn, may increase glucose uptake via either the Ras-PI3K pathway or another unknown mechanism. To clarify this further, future work...
A. Ad-36 increases glucose uptake in human skeletal muscle cells

in vitro (Fig. 7). Ad-2 does not increase RAS abundance or glucose uptake in animals and humans, respectively (33, 61). We showed that Ad-36 selectively overexpresses Ras in adipose tissue in vivo and increases glucose uptake by adipose tissue. This assertion is supported by an earlier study (52) that showed that selective overexpression of Ras in adipose tissue increased glucose uptake by the tissue and improved whole-body insulin sensitivity of the transgenic mice.

Although like Ad-36, other human adenoviruses activate Ras and PI3K signaling pathways, which may then increase glucose uptake in vitro, their ability to modulate the metabolic profile of adipose tissue is unknown. For instance, unlike Ad-36, human adenoviruses Ad-2 and Ad-31 do not show causation or association with adiposity in animals and humans, respectively (33, 61). We showed that Ad-2 does not increase RAS abundance or glucose uptake in vitro (Fig. 7C and D). Ad-9 upregulates PI3K via Ras signaling (47) and enhances adipogenesis in vitro (22), but its effect on glucose disposal is unknown. Characteristics unique to Ad-36 that help it increase adipogenesis and glucose uptake are unclear.

Evolution of human adenoviruses on proinflammatory cytokines is varied. Ad-36 reduced MCP-1 expression in explants, but Ad-19 increases MCP-1 expression (62) and Ad-7 increases interleukin-8 production (63). On the other hand, adenovirus types 16, 35, and 37 reduce proinflammatory cytokine expressions (64). Interestingly, Ad-37 is reported to increase adiposity in animals (33). Overall, unlike Ad-36, other adenoviruses have not been tested comprehensively for their effects on adipogenic pathways and glucose metabolism. Recognizing the ability of Ad-36 to modulate metabolic profile of adipose tissue should provide impetus to evaluate other human adenoviruses for similar potential.

Increased adiposity is associated with lower adiponectin levels and greater inflammation and insulin resistance, and reduction in adipose tissue mass reverses the changes (65–69). However, reduction of adipose tissue and preventing its regain are challenging. Our findings indicate that akin to some therapeutic agents (5, 11), it is possible to induce a metabolically favorable profile in the adipose tissue, without a reduction in adiposity. This is a potentially important finding for eventually developing novel strategies to manage adiposity-induced glucose dysregulation. Further studies are required to identify the viral protein responsible for the effect and to elucidate its interaction with cellular proteins participating in tissue remodeling.

Finally, the association of natural Ad-36 infection with better glycemic control in diabetic and nondiabetic human subjects provides a proof of concept that is expected to offer a human relevance to the main observations described. The number of Ad-36 seropositive subjects is relatively small in these studies, and a dedicated larger prospective study with human samples better characterized for glucose metabolism is required to further test the association. Nevertheless, it is interesting that even with a small number of Ad-36–positive subjects, this study conducted in a blinded manner yielded 40% lower glucose values compared with Ad-36–negative subjects (Table 1A).

B. Ad-36 seropositivity in diabetic subjects

| Measure                        | Ad-36 positive | Ad-36 negative | P    |
|-------------------------------|----------------|----------------|------|
| Age (years)                   | 67 ± 10        | 60 ± 17        | NS   |
| BMI (kg/m²)                   | 34.6 ± 5       | 35.9 ± 6       | NS   |
| Waist circumference (cm)      | 106.2 ± 9      | 111.6 ± 14     | NS   |
| Weight (kg)                   | 88 ± 9         | 98 ± 16        | NS   |
| Percent body fat (%)          | 42.3 ± 4       | 39.5 ± 9       | NS   |
| Visceral adipose tissue (kg)  | 6.0 ± 0.6      | 6.7 ± 2        | NS   |
| Systolic blood pressure (mmHg)| 130 ± 17       | 125 ± 16       | NS   |
| Diastolic blood pressure (mmHg)| 79 ± 8        | 78 ± 9         | NS   |
| Total cholesterol (mg/dl)     | 167 ± 31       | 199 ± 34       | 0.02 |
| LDL cholesterol (mg/dl)       | 78 ± 37        | 111 ± 27       | 0.04 |
| A1C (%)                       | 5.7 ± 0.4      | 6.3 ± 0.8      | 0.005|

Data are means ± SD unless otherwise indicated.

should determine whether Ras or PI3K knockdown will attenuate Ad-36–induced glucose uptake, when the viral gene or protein expression is maintained by a different promoter.

Human adenoviruses are known to cooperate with Ras in cell transformation (59) and to activate PI3K via Ras signaling (47) and increase glucose uptake in vitro (60). As predicted, Ras was required for Ad-36–induced activation of PI3K and the consequent increase in glucose uptake in basal as well as insulin-stimulated glucose uptake. Thus, Ras signaling appears to be a key event in Ad-36–induced increase in glucose uptake by adipose tissue. This assertion is supported by an earlier study (52) that showed that selective overexpression of Ras in adipose tissue increased glucose uptake by the tissue and improved whole-body insulin sensitivity of the transgenic mice.

TABLE 1
Association of Ad-36 infection with glycemic control in humans

- Ad-36 positive
- Ad-36 negative
- P

A. Ad-36 increases glucose uptake in human skeletal muscle cells

n(%) 5 (13.5) 32 (86.5) NS
Age (years) 46.7 ± 11 49.1 ± 9 NS
BMI (kg/m²) 43.6 ± 4.6 47.4 ± 5.3 NS
Systolic blood pressure (mmHg) 125 ± 17 129 ± 18 NS
Diastolic blood pressure (mmHg) 79 ± 14 82 ± 10 NS
Glucose (mg/dl) 74 ± 29 124 ± 53 0.02

B. Ad-36 seropositivity in diabetic subjects

n(%) 6 (12.5) 42 (87.5) NS
Age (years) 67 ± 10 60 ± 17 NS
BMI (kg/m²) 34.6 ± 5 35.9 ± 6 NS
Waist circumference (cm) 106.2 ± 9 111.6 ± 14 NS
Weight (kg) 88 ± 9 98 ± 16 NS
Percent body fat (%) 42.3 ± 4 39.5 ± 9 NS
Visceral adipose tissue (kg) 6.0 ± 0.6 6.7 ± 2 NS
Systolic blood pressure (mmHg) 130 ± 17 125 ± 16 NS
Diastolic blood pressure (mmHg) 79 ± 8 78 ± 9 NS
Total cholesterol (mg/dl) 167 ± 31 199 ± 34 0.02
LDL cholesterol (mg/dl) 78 ± 37 111 ± 27 0.04
A1C (%) 5.7 ± 0.4 6.3 ± 0.8 0.005

Better glycemic control in Ad-36–infected animals was discovered recently (18). Therefore, a seropositivity-dependent difference, if any, in glucose values was not determined in our earlier study that showed Ad-36 seropositivity in 30% of obese and 11% of the nonobese subjects screened (70). We revisited unpublished data of this study (R.L. Attinson, B.A. Israel, A.S. Augustus, N.V. Dhurondhor, unpublished data). Fasting glucose measurements were available for 85 obese subjects (73 seronegative and 12 seropositive for Ad-36) recruited
from Wisconsin. The fasting blood glucose levels were lower for the Ad-36–seropositive group (102 ± 36 vs. 95 ± 7.6; \( P = 0.06 \)).

Better glycemic control in Ad-36–infected subjects is intriguing. Ad-36–neutralizing antibody titer was not high enough to suggest an acute infection. This indicates a longer-lasting change in systemic glucose handling following a natural infection in these subjects. It is noteworthy that Ad-36 increases adiposity in experimentally infected animals and improves glycemic control, but only the later was observed to be associated with Ad-36 infection in these subjects. Considering the numerous other adipogenic stimuli in humans, it is possible that the Ad-36–negative subjects gained adiposity due to other causes. We postulate that the Ad-36–induced expansion of adipose tissue is accompanied by improvement in metabolic profile of the tissue, compared with a similar degree of adiposity acquired due to other reasons.

Although Ad36 remarkably increases adipose tissue–glucose uptake independent of insulin in vitro, the virus does not cause uncontrolled glucose uptake and hypoglycemia in experimentally infected rats (18) or humans naturally infected with Ad-36. Perhaps this is because the in vivo physiological regulatory controls of circulating glucose homeostatic mechanisms prevent uninhibited glucose clearance and consequential fasting hypoglycemia. Moreover, Ad-36 induces glucose uptake in a virus dose–dependent manner (37). The degree of infectivity may be another determinant of the magnitude of glucose uptake induced by the virus in vivo. It is likely that due to immune response, tissue accessibility, or other factors, a relatively limited fraction of cells are infected in an organism. Hence, the net result of contributions from infected and uninfected cells may balance out the robust effect of Ad-36 on cellular glucose uptake observed in vitro to a more moderate effect without severe hypoglycemia in vivo.

We previously reported that Ad-36 increases the commitment of hASCs to adipogenic lineage (71). Moreover, similar to its effect on adipose tissue, Ad-36 also decreases glucose uptake by human skeletal muscle (37) in a Ras-mediated, PI3K-dependent manner. Ad-36 increases FAS levels, suggesting the conversion of increased cellular glucose to lipids via de novo lipogenic pathway. Taken together, we postulate that Ad-36 expands adipose tissue and increases glucose uptake in skeletal muscle and adipose tissue, which collectively leads to better glycemic control in vivo. By further identifying the viral proteins and their cellular targets involved in the effect, novel therapeutic agents may be developed for enhancing glucose disposal in type 1 or type 2 diabetes and for improving the adipose tissue metabolic profile associated with insulin resistance.

ACKNOWLEDGMENTS

This study was supported in part by funds from the National Institutes of Health (NIH R-01DK066164 [to N.V.D.] and P50AT002776-01 and R01DK060126 [to W.T.C.]). We gratefully acknowledge that human adipose tissue and isolated hASCs were provided by Dr. Jeffery M. Gimble of the Molecular Mechanisms Core of the Pennington Biomedical Research Center Clinical Nutrition Research Unit (NIH 1P30DK072476).

REFERENCES

1. Oh DK, Ciaraldi T, Henry RR: Adiponectin in health and disease. Diabetes Obes Metab 9:282–289, 2007
2. Bastard JP, Lagathu C, Maachi M, et al.: [Adipose tissue cytokines and insulin resistance]. J Am J Diabetol 29–37, 2004 [Article in French]
3. Capeau J, Magre J, Lascols O, et al.: Diseases of adipose tissue: genetic and acquired lipidostathies. Biochem Soc Trans 33:1073–1077, 2005
4. Eganathan G, Unal R, Pekovskaia I, et al.: The lipogenic enzymes DGAT1, FAS, and LPL in adipose tissue: effects of obesity, insulin resistance, and T2D treatment. J Lipid Res 47:2444–2450, 2006
5. de Souza CJ, Eckhardt M, Gagen K, et al.: Effects of pioglitazone on adipose tissue remodeling within the setting of obesity and insulin resistance. Diabetes 50:1863–1871, 2001
6. Tarcin O, Bajaj M, Akalin S: Insulin resistance, adipocyte biology and thiazolidinediones: a review. Metab Syndr Relat Disord 5:103–115, 2007
7. Standaert ML, Koenig K, Tanabe MP, et al.: Cbl, IRS-1, and IRS-2 mediate effects of rosiglitazone on PI3K, PDK-1, and glucose transport in 3T3/L1 adipocytes. Endocrinology 143:1706–1716, 2002
8. Todd MK, Watt MJ, Le J, et al.: Thiazolidinediones enhance skeletal muscle triacylglycerol synthesis while protecting against fatty acid-induced inflammation and insulin resistance. Am J Physiol Endocrinol Metab 292:E485–E492, 2007
9. van Doorn M, Kemme M, Ouwen M, et al.: Evaluation of proinflammatory cytokines and inflammation markers as biomarkers for the action of thiazolidinediones in type 2 diabetes mellitus patients and healthy volunteers. Br J Clin Pharmacol 62:391–402, 2006
10. Yu JG, Javorschi S, Hevener AL, et al.: The effect of thiazolidinediones on plasma adiponectin levels in normal, obese, and type 2 diabetic subjects. Diabetes 51:2968–2974, 2002
11. Fung Y, Liu X, et al.: Effect of a novel non-thiazolidinedione peroxisome proliferator-activated receptor alpha/gamma agonist on glucose uptake. Diabetol 29:1048–1057, 2007
12. Smith SR, Xie H, Baghian S, et al.: Pioglitazone changes the distribution of adipocyte size in type 2 diabetes. Adipocytes 211:22–66, 2006
13. Medina-Gomez G, Gray SL, Yetukuri L, et al.: PPAR gamma 2 prevents lipotoxicity by controlling adipose tissue expandability and peripheral lipid metabolism. PLoS Genet 3:e64, 2007
14. Kim JY, van de Wall E, Lapham M, et al.: Obesity-associated improvements in metabolic profile through expansion of adipose tissue. J Clin Invest 117:2621–2637, 2007
15. Dhurandhar NV, Israel BA, Kolesar JM, et al.: Transmissibility of adenovirus-induced adiposity in a chicken model. Int J Obes Relat Metab Disord 25:990–996, 2001
16. Dhurandhar NV, Israel BA, Kolesar JM, et al.: Increased adiposity in animals due to a human virus. Int J Obes Relat Metab Disord 24:989–996, 2000
17. Dhurandhar NV, Whigham LD, Abbott DH, et al.: Human adenovirus Ad-36 promotes weight gain in rhesus macaques and marmoset monkeys. J Nutr 132:3155–3160, 2002
18. Pasarica M, Shin AC, Yu M, et al.: Human adenovirus 36 induces adiposity, increases insulin sensitivity, and alters hypothalamic monoamines in rats. Obesity (Silver Spring) 14:1905–1913, 2006
19. Angphupum SD, Scheele J, Atkinson RL, et al.: A human adenovirus enhances preadipocyte differentiation. Obes Res 12:770–777, 2004
20. Rathod M, Angphupum SD, Krishnan B, et al.: Viral mRNA expression but not DNA replication is required for lipogenic effect of human adenovirus Ad-36 in preadipocytes. Int J Obes Relat Metab Disord 25:809–816, 2001
21. Rathod MA, Rogers PM, Dhurandhar NV: Human adenovirus Ad-36 infection induces differentiation and replication of preadipocytes. Obes Res 7:138, 2006
22. Angphupum SD, Yu M, Tian J, et al.: Adipogenic human adenovirus-36 reduces leptin expression and secretion and increases glucose uptake by fat cells. Int J Obes (Lond) 31:87–96, 2007
23. Rogers PM, Fusinski K, Rathod MA, et al.: Human adenovirus Ad-36 induces adipogenesis via its E4 orf-1 gene. Int J Obes 32:397–406, 2007
24. Hanlon GW: Bacteriophages: an appraisal of their role in the treatment of bacterial infections. Int J Antimicrob Agents 30:118–128, 2007
25. Bischoff JR, Kin Dh, Williams A, et al.: An adenovirus mutant that replicates selectively in p53-deficient human tumor cells. Science 274:373–376, 1996
26. Crompton AM, Kinn Dh: From ONX-015 to armed vaccinia viruses: the education and evolution of oncolytic virus development. Curr Cancer Drug Targets 7:133–139, 2007
27. Pan Q, Liu B, Liu J, et al.: Synergistic induction of tumor cell death by
