Metabolically Favorable Remodeling of Human Adipose Tissue by Human Adenovirus Ad-36

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Key words: Infectobesity, insulin resistance, adipose tissue, metabolic remodeling, glucose uptake, Ad-36

Received 13 September 2007 and accepted 11 June 2008.

Additional information for this article can be found in an online appendix at http://diabetes.diabetesjournals.org

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Ad-36 increases glucose uptake in human skeletal muscle cells

Objectives: Experimental infection of rats with a 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 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 if 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 adipose derived stem cells (hASC). Ad-2, a non-adipogenic human adenovirus was used as a negative control. As a proof of concept, non-diabetic and diabetic humans 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 non-diabetic and diabetic subjects. Without insulin stimulation, Ad-36 up-regulated expressions of several pro-adipogenic genes, adiponectin and fatty-acid-synthase (FAS), and reduced the expression of inflammatory cytokine – macrophage-chemoattractant-protein-1 (MCP-1), in a phosphotidyl-inositol 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 non-adipogenic and did not increase glucose uptake. Natural Ad-36 infection in non-diabetic and diabetic human subjects was associated with significantly lower fasting glucose levels and HbA1c, respectively.

Conclusion: Ad-36 proteins may provide novel therapeutic targets that remodel human adipose tissue to a more metabolically favorable profile.
Obesity is associated with adverse metabolic profile of adipose tissue, including impaired adipogenesis, lower FAS and adiponectin, and increased secretion of inflammatory cytokines. Consequently, this contributes to increase in insulin resistance and a reduction in glucose uptake by the tissue(1-3)(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, thiazolidinediones (TZD) class of drugs increase glucose uptake in response to insulin stimulation(5), induce PPARγ2 and increase adipogenesis(6), activate phosphotidylinositol 3-kinase (PI3K)(7), reduce the release of inflammatory cytokines(8)(9), and up-regulate adiponectin secretion(10) and fatty acid synthase (FAS) expression(4) in the 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(12)(5), and offsetting 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(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 over 60%, but reduced the fasting insulin levels and HOMA index by about 50% for up to 7 months later(18), a robust and long-term effect that is reminiscent of the TZDs. Moreover, Ad-36 up-regulates 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 had a recent resurgence in interest (Review-(24)). Furthermore, the first report of oncolytic ability of a mutant adenovirus(25) was followed by numerous studies extending the use of oncolytic properties of various viruses. Herpes simplex virus, Newcastle disease virus, reovirus and vaccinia virus were used for the treatment of various cancers (Review–(26)), alone, or in combination of various synergistic drugs(27)(28). The potential of Ad-36 proteins to enhance glucose disposal and improve adipose tissue metabolic profile could be exploited as therapeutic targets for humans, a natural host of the virus. To investigate this possibility, we first determined if Ad-36 increases glucose uptake in primary adipose tissue explants obtained from healthy and diabetic subjects. Next, we elucidated the cellular signaling pathways involved in Ad-36 induced increase in glucose uptake. Finally, as a proof of concept, non-diabetic and
diabetic humans 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.

MATERIALS AND METHODS

Human adipose tissue samples were obtained from 9 healthy, non-diabetic female patients and 1 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, age 28-50y (mean=39+9.5y), BMI 19.5-28.2kg/M² (mean=23.7+3.3). Small chunks of adipose tissue were cultured as explants, as described below and used to elucidate the effect of Ad-36 infection. hASC were obtained from subjects with BMI 25-30kg/M² and age 47-58y. At least three or more technical replicates were used for all experiments. Sample from two or more subjects were used for Ad-36 experiments, except for the diabetic sample. Effect of Ad-2 infection was tested in adipose tissue and hASC of two separate individuals. An outline of the experiments is presented below, followed by a detailed description of the techniques and assays.

a. 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 non-diabetic and diabetic subjects. Adipogenesis was assessed by determining time course of expressions of adipogenic genes: PPARγ2, aP2 (fatty acid binding protein), lipoprotein lipase (LPL), and glycerol 3-phosphate dehydrogenase (G3PDH), and by counting adipocytes. Considering their important roles in glycemic control, activation of PI3K, and gene expression and protein abundance of adiponectin and FAS in adipose issue explants were determined. The gene expression assays conducted for Ad-36 infection were repeated and compared in Ad-2 vs mock infected adipose tissue explants.

b. Requirement of PI3K activation for Ad-36 induced alteration in metabolic profile: Requirement of PI3K in Ad-36 induced metabolically favorable remodeling of adipose tissue was determined by using Wortmainnin (WM), a specific inhibitor of PI3K activity. The effect of WM on expressions of selected cellular genes (PPARγ2, aP2, LPL, PECAM-1 (Platelet-endothelial Cell Adhesion Molecule – as a marker for angiogenesis induction) and MCP-1 (macrophage-chemoattractant-protein-1 – a marker of pro-inflammatory cytokine), adiponectin, FAS, and E4orf-1 – a viral gene, was determined in adipose tissue explants infected with Ad-36 or mock controls. Finally, the effect of WM on glucose uptake by the explants was determined.

c. Role of Ras and insulin receptor signaling in activation of Ad-36 induced PI3k activation: Roles of two major signaling pathways in Ad-36 induced PI3K activation were determined. Phosphorylations of insulin receptor (IR), insulin receptor substrate (IRS)-1 and IRS-2, and protein abundance of Ras were compared in explants infected with Ad-36 or mock infected. Additional experiments were conducted in hASC to better elucidate the effect of Ad-36 on signaling pathways. The role of Ad-36 in activation of IRS-1 and PI3K, was determined with and without stimulation by insulin, a well known activator of the pathway. Finally, the requirement of Ras, another key activator of PI3K, was determined in Ad-36 induced PI3K activation and glucose uptake by hASC. Effect of Ras knockdown on PI3K pathway activation and glucose uptake was also determined in Ad-2 infected hASC.

d. 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 non-diabetic 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). Serum 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 HbA1c 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.** Prior to infection, explants (~100mg for gene expression assays, ~20mg for glucose uptake) were cultured for 3days in DME/F-12 (Hyclone #SH30023.01) 1:1 +20% FBS (Hyclone #SH30070.03) +antibiotic/antimycotic at 37°C in 5% CO₂ unless otherwise indicated. For PI3K inhibition, 0.01μM Wortmannin (Sigma #W1628) was added to cultures post inoculation. Adipose tissue explants dedifferentiate 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 isobutyl methyl xanthine (IBMX)(29). We assessed this in two separate preliminary experiments (Supplementary Figure 1 A-B). First, we cultured explants up to 15days to determine the drop in gene expression and observed stabilization of PPARγ mRNA expression after 3 days. In the second experiment, we verified if the addition of 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 0.1mM IBMX (Sigma #I5879)(29). Media was replaced every 2days. 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 about 3 days after initiating incubation.

**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 +10% FBS+antibiotic/antimycotic and plated at a density of 0.156 mL of tissue digest/cm² as passage 0 (p0) and used for experiments within passages 2 to 4 (p2-p4). No Ad-36 DNA was detected in these tissues prior to experimentation.

**Virus preparation.** Ad-36 was obtained from American Type Culture collection (ATCC Cat# VR913) 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 ATCC (Cat #VR846) and propagated in A549 cells. Viral titers were determined by plaque assay(16) and cell inoculations expressed as plaque forming units (PFU)/cell, unless otherwise indicated.

**Ad-36 or Ad-2 infection of human adipose tissue explants.** Explants were incubated for 1h with 100μL/cm² DME/F-12 1:1 (Mock) or 100μL/cm² DME/F-12 1:1 +Ad-36 or Ad-2 (10⁶ infectious particles). Following the infection, media and virus were removed and replaced with DME/F-12 1:1 + 10%FBS + antibiotic/antimycotic.

**Infection of hASCs.** hASC cultures were seeded at 15000 cells/cm². At confluency, culture media was removed and cells incubated for 1hr with 100μL/cm² DME/F-12 1:1 (Mock), DME/F-12 1:1 +Ad-36 or Ad-2 (2.7 PFU/cell, unless noted otherwise). Following infection, media and virus were removed and replaced with DME/F-12 1:1 +20% FBS + antimycotic/antibiotic.

**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 (AU).
Priming for qRT-PCR. Primers for human PECAM-1 were purchased from Applied Biosystems. All other primer sequences were as follows.

Ad-36E4orf-1 forward: 5'-GGCATACTAACCAGTGCCATG-3'
Ad-36E4orf-1 reverse: 5'-AATACACTCTCCAGCACAGG-3'

Ad-2E4orf-1 forward: 5'-CCTAGGCCAGGAGGTTTTC-3'
Ad-2E4orf-1 reverse: 5'-ATAGCCGGGGGAATACATA-3'

human cyclophilinB forward: 5'-GGAGATGGCACAGGAGGAAA-3'
human cyclophilinB reverse: 5'-CGTAGTGCTTCAGTTTGAAGTTCTCA-3'

human PPARγ2 forward: 5'-GATACACTGTCTGCAAAACATATCACAA
human PPARγ2 reverse: 5'-CCACGGAGCTGATCCCAA-3'

human LPL forward: 5'-TATCCGCGTGATTGCAGAGA-3'
human LPL reverse: 5'-AGAGAGTCGATGAAGAGATGAATGG-3'

human aP2 forward: 5'-TGGTTGATTTTCCATCCCAT-3'
human aP2 reverse: 5'-TACTGGGCCAGGAATTTGAT-3'

human GPDH forward: 5'-CTATACACTGTCTCAGAGAKCAACAA-3'
human GPDH reverse: 5'-GGCCCTCGTAGACATCCTCA-3'

human adiponectin forward: 5'-TCTGTTCCTCCACCTCCTGTGA-3'
human adiponectin reverse: 5'-CAGGCCCGTCATCATAGAACCACCT-3'

human FAS forward: 5'-TATGCTTCTTCTGCGACAGTGT-3'
human FAS reverse: 5'-GCTGCCACACGCTCTCTTAG-3'

human MCP-1 forward: 5'-CAGCCAGATGCAATCAATGC-3'

Western blot analysis. Protein concentrations were quantitated by BCA assay and loaded to the 4%-20% or 10%PAAG in equal amounts. Proteins then were transferred to PVDF membrane. Membranes were blocked in PBST 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 PKB, total PKB, Ras, secondary antibodies and α/β-tubulin were purchased from Cell Signaling (Danvers, MA, USA). Antibodies to adiponectin and FAS were purchased from Abcam (Cambridge, MA, USA). Antibodies to IRβ were purchased from Upstate (Chicago, IL, USA). Antibodies to IRS-1, IRS-2 were purchased from Santa Cruz (Santa Cruz, CA, USA).

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 2h and placed in 70% ethanol overnight prior to processing. Tissues were embedded in paraffin and 6μm slices subjected to standard 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 post infection by Western blotting as described above. Adiponectin levels in the media were normalized to total protein of explants and expressed as AU.

Glucose uptake assays. D-[3H]-deoxyglucose was purchased from Amersham (Piscataway, NJ, USA). Glucose uptake assays were performed as previously described(31). Briefly, human adipose tissue...
explants (~20mg) were incubated at 37°C, 5%CO₂ for 30 minutes in 500μL DME/F-12 1:1 +2% BSA. In a pilot experiment, we determined the glucose uptake by adipose tissue explants to be linear up to 40 min of incubation (Supplementary Figure 2). For adequate tissue permeability, 30 min incubation was used. Explants were washed three times in Krebs-Ringer HEPES (KRH) buffer+1% BSA. Explants were incubated for 15 minutes in KRH buffer+1% BSA prior to the addition of 2 μM [³H]-D-deoxy-glucose and 50μM glucose to each well and further incubation for 30 minutes. Explants were washed 3X in ice-cold KRH+1% BSA to stop the reaction and to remove unincorporated label. Explants were blotted and incubated for 30 minutes at 65°C in 1N NaOH prior to protein quantitation and 3H radioactivity was determined by scintillation counting. Data are expressed as pmol 2-deoxyglucose/mg protein.

Ras shRNA assay. hASC cells were cultured to 80% confluency in growth media in 100mm dishes and infected with Ad-36 (3.8 PFU/cell). Four days post infection, Ras shRNA vector transfection was conducted using 20 μg pKD-Ras-v1 plasmid (Cat#62-214) or pKD-neg control-v1 plasmid (Cat#62-002) from Upstate Biotechnology (Lake Placid, NY). The efficiency of Ras knockdown was confirmed by qRT-PCR. Four days post shRNA transfection, glucose uptake was determined in mock or Ad-36 infected cells, Ad-36 infected cells transfected with Ras shRNA or negative control shRNA, without insulin or after 100nM insulin stimulation for 15 min. Effect of Ras knockdown on glucose uptake and PI3K activity was determined.

In a separate experiment, hASC 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 post infection, glucose uptake was determined and the samples were harvested for western blot analysis.

PI3K activity assay: 500 μg of hASC total protein extract was subjected to immunoprecipitation with 3 μg of PI3K p85 polyclonal antiserum (Upstate) to determine PI3K activity as previously described(19). The PI3K-phospholipid product was visualized by autoradiography, and quantitated by scanning densitometry with Quantity one 1D software version 4.2.1 using Bio-Rad gel documentation.

Infectivity of hASC: Near confluent hASC were infected with serial 10-fold dilutions of 100μL stock of viruses Ad-36 and Ad-2 (triplicate for each dilution) and overlaid with agar. Starting PFU (calculated using A5349 cells) was 6.5X10⁷ /ml; and 8X10⁸/mL for Ad-36 and Ad-2, respectively. Cells were fixed 8 days post infection and stained with DAPI (4’,6-diamidino-2-phenylindole dihydrochloride, Invitrogen; cat#D1206), and adenovirus hexon antibodies (rabbit antiserum, American Type Culture Collection cat#VR1079) followed by Alexa-fluor-594 goat anti-rabbit antibodies, (Invitrogen; cat#A-11012). Number of plaques formed (as evident by cells expressing viral proteins) by Ad-36 and Ad2 were compared for dilutions that used similar PFU to infect (8 x 10³ PFU for Ad-2 and 6.5 x 10³ PFU for Ad-36).

Statistics. Assays were performed in triplicate and reported as Mean±SEM. 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 Ad-2 infected hASC by two-way ANOVA followed by Tukey-Kramer test. Analyses were conducted using functions available in the base package of R(32) or SAS.

RESULTS

a. Ad-36 improves the metabolic profile of adipose tissue: Presence of Ad-36 E4orf-1 gene expression and its time-dependent increase confirmed successful viral entry in cells and its spread in the tissue (Figure 1A). The viral infection increased PI3K activation in the adipose tissue, as indicated by PKB phosphorylation (Figure 1B), which probably contributed to increased glucose uptake in the adipose tissue of non-diabetic and diabetic subjects (Figure 1C & D). Ad-36 induced the expression of early (PPARγ2) and late (LPL, aP2, G3PDH) genes of adipogenic cascade, which was maintained over 15 days post inoculation (Figure 2A-D).
Expressions of these genes did not change in the mock infected groups. As suggested by activation of the adipogenic cascade, Ad-36 infected explants significantly increased the number of adipocytes by 21 d post inoculation (Figure 2 E-G). Ad-36 infected adipose tissue also showed greater mRNA expression and protein abundance of adiponectin and FAS (Figures 3A-D). Thus, in human adipose tissue, Ad-36 activates adipogenesis, enhances FAS and adiponectin and increases glucose disposal, indicating improved metabolic profile of the tissue.

The effect of Ad-36 on adipose tissue can not be attributed simply to any viral infection. Ad-2, another human adenovirus, which is non-adipogenic in-vivo and in-vitro (23; 33), was used as a negative control. In addition, we conducted plaque forming units assay in hASC. The number of plaques formed in hASC by nearly equal number of particles of the two viruses were not significantly different (Ad-36 vs Ad-2, Mean+SD 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 hASC. Despite successful infection of the explants as evident from viral mRNA expression, (Figure 4A), unlike Ad-36, Ad-2 did not induce expression of the above-described genes of adipogenic cascade (Figures 4 B-G). Therefore, subsequently, we focused only on the effect of Ad-36.

b. 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 WM reduced the expressions of aP2, PECAM-1, adiponectin, FAS and MCP-1 (Figure 5A). Also, Ad-36 E4orf-1 gene expression requires PI3K activation (Figure 5B). Furthermore, Ad-36 induced expression of PPARγ2, aP2, LPL, PECAM-1 in the adipose tissue was dependent on PI3K activation (Figures 5C-F). Via PI3K activation, Ad-36 suppressed expression of MCP-1, a pro-inflammatory marker. WM reversed Ad-36 induced MCP-1 suppression, which also showed that its effect on cellular gene expression is not universally inhibitory in presence of the virus. Importantly, Ad-36 required PI3K to enhance glucose uptake (Figure 5G). Collectively, these results demonstrated that Ad-36 induces metabolic changes in adipose tissue via activation of PI3K.

c. 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 hASC. Ad-36 reduced the abundance and activation of IR, IRS-1 and IRS-2 in explants, but greatly increased Ras protein abundance in explants (Figure 6A), suggesting that the virus uses Ras signaling, rather than insulin receptor signaling for PI3K activation. In hASC, under basal or insulin stimulated conditions, Ad-36 activated PKB phosphorylation, but blocked IRS-1 tyrosine-phosphorylation (Figures 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 PI3K pathway. As hypothesized, Ras specific shRNA abrogated Ad-36 induced PI3K activity (Figure 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 hASC is Ras dependent (Figure 7B). Interestingly, Ad-36 induced 4-fold increase in glucose uptake by hASC, which could not further be enhanced by insulin stimulation (Figure 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 hASC. Compared to the mock infected hASCs, Ad-36, but not Ad-2, increased glucose uptake by about 2.5-fold (Figure 7C), Ras abundance knockdown was up to 50%
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(Figure 7D). As expected, 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 (Figure 7D), Ad-2 did not increase Ras abundance, PKB activation or glucose uptake. Ad-2 protein abundance was not affected by Ras knockdown.

d. Natural infection of Ad-36 is associated with better glycemic control in humans:
Blinded post hoc screening for neutralizing antibodies to Ad-36 in non-diabetic as well as diabetic subjects enrolled in unrelated studies showed about 12% prevalence of Ad-36 infection (Tables 1A-B). Study 1 included 3 men and 34 women who were non-diabetic or diabetic, (31 Caucasian and 6 African Americans) (Table 1A). Study 2 included 16 men and 32 women 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, supplementary Figure 3) or HbA1C levels (Table 1B; supplementary Figure 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 comprises of various cell types, including those of adipogenic, endothelial and of 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 to test the potential of Ad-36 to favorably alter the metabolic profile of the entire tissue, including the pro-adipogenic, 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, hASC were used to further elucidate molecular mechanism.

Overall, Ad-36 improves metabolic profile of human adipose tissue as indicated by greater glucose uptake, reduced expression of MCP-1 - a pro-inflammatory marker, increased FAS and adiponectin levels and the increased expression of adipogenic genes such as PPARY2, 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 (Figure 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 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. Pivotal role of PI3K in cellular metabolism was also evident from the reduction in cellular gene expression in presence of WM (Figure 5 A-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(46; 47)(45; 48). Since the adipose tissue was infected with Ad-36 prior to WM treatment, 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 insulin receptor activates insulin receptor's internal tyrosine kinase activity. The activated tyrosine-phosphorylated insulin receptor phosphorlates 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 hASC, perhaps due to a negative feedback from activated PI3K as shown by(53) and reviewed by(54). Therefore, enhanced activation of PI3K signaling by insulin in 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), who showed that tyrosine phosphorylation of insulin receptor (IR) by insulin activates Ras via SOS, a guanine nucleotide exchange protein, and such activation is independent of IRS-1 activation.

Ad36-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 Ad36 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. Ad36 may upregulate 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 should determine if Ras or PI3K knockdown will attenuate Ad36-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 transforming(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 consequential 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, which 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(52).

Although like Ad-36, other human adenoviruses activate Ras and PI3K signaling pathways, which may then increases glucose uptake in-vitro, their ability to modulate 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 (Figure 7C-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.

Effect of human adenoviruses on pro-inflamatory cytokines is varied. Ad-36 reduced MCP-1 expression in explants, but Ad-19 increases MCP-1 expression(62) and Ad-7 increases IL-8 production(63). Whereas, 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
Ad-36 increases glucose uptake in human skeletal muscle cells

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, greater inflammation and insulin resistance and reduction in adipose tissue mass reverses the changes(65-67)(68; 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 disregulation. 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 non-diabetic human subjects provides a proof of concept, which is expected to offer a human relevance to the main observations described. The number of Ad36 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 Ad36 positive subjects, this study conducted in a blinded manner yielded a 40% lower glucose levels (Table 1A), 16% lower serum cholesterol, 30% lower LDL-cholesterol, and 10% lower HbA1c levels (Table 1B) in Ad36 seropositive groups.

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 the obese and 11% of the non-obese subjects screened (70). We re-visited unpublished data of this study. Fasting glucose measurements were available for 85 obese subjects (73 seronegative, 12 seropositive for Ad36) recruited from Wisconsin. The fasting blood glucose levels were lower for the Ad36 seropositive group (102±36 vs 95±7.6; p=.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 Ad36 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 Ad36-negative subjects gained adiposity due to other causes. We postulate that the Ad36-induced expansion of the adipose tissue is accompanied by improvement in metabolic profile of the tissue, compared to 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 virus-dose dependent manner(37). Therefore, 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 Ad36 on cellular glucose uptake observed in-vitro to a more moderate effect without severe hypoglycemia in-vivo.
We previously reported that Ad-36 increases commitment of hASC to adipogenic lineage\(^{(71)}\). Moreover, similar to its effect on adipose tissue, Ad-36 also increases 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 \textit{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 \textit{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 adipose tissue metabolic profile associated with insulin resistance.

**ACKNOWLEDGMENTS**

Supported in part by funds from NIH R-01DK066164 awarded to NVD and P50AT002776-01 and R01DK060126 awarded to WTC. We gratefully acknowledge that human adipose tissue and isolated hASC were provided by Dr. Jeffery M. Gimble of the Molecular Mechanisms Core of the Pennington Biomedical Research Center Clinical Nutrition Research Unit (NIH 1P30DK072476).
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Table 1: Association of Ad-36 infection with glycemic control in humans

A  Ad-36 seropositivity in diabetic and non-diabetic subjects

<table>
<thead>
<tr>
<th></th>
<th>Ad-36 Positive (N = 5, 13.5%)</th>
<th>Ad-36 Negative (N = 32, 86.5%)</th>
<th>p-value</th>
</tr>
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<tbody>
<tr>
<td>Age (years)</td>
<td>46.7±11</td>
<td>49.1±9</td>
<td>NS</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>43.6±4.6</td>
<td>47.4±5.3</td>
<td>NS</td>
</tr>
<tr>
<td>SBP (mm/Hg)</td>
<td>125±17</td>
<td>129±18</td>
<td>NS</td>
</tr>
<tr>
<td>DBP (mm/Hg)</td>
<td>79±14</td>
<td>82±10</td>
<td>NS</td>
</tr>
<tr>
<td>Glucose (mg/dL)</td>
<td>74±29</td>
<td>124±53</td>
<td>0.02</td>
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</table>

B  Ad-36 seropositivity in diabetic subjects

<table>
<thead>
<tr>
<th></th>
<th>Ad-36 Positive (N = 6, 12.5%)</th>
<th>Ad-36 Negative (N = 42, 87.5%)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>67±10</td>
<td>60±17</td>
<td>NS</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>34.6±5</td>
<td>35.9±6</td>
<td>NS</td>
</tr>
<tr>
<td>Waist Circumference (cm)</td>
<td>106.2±9</td>
<td>111.6±14</td>
<td>NS</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>88±9</td>
<td>98±16</td>
<td>NS</td>
</tr>
<tr>
<td>Percent Body Fat (%)</td>
<td>42.3±4</td>
<td>39.5±9</td>
<td>NS</td>
</tr>
<tr>
<td>Visceral adipose tissue (kg)</td>
<td>6.0±0.6</td>
<td>6.7±2</td>
<td>NS</td>
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<tr>
<td>Systolic BP (mm of Hg)</td>
<td>130±17</td>
<td>125±16</td>
<td>NS</td>
</tr>
<tr>
<td>Diastolic BP (mm of Hg)</td>
<td>79±8</td>
<td>78±9</td>
<td>NS</td>
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<tr>
<td>Total-Cholesterol (mg/dL)</td>
<td>167±31</td>
<td>199±34</td>
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</tr>
<tr>
<td>LDL-Cholesterol (mg/dL)</td>
<td>78±37</td>
<td>111±27</td>
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<tr>
<td>HbA1c (%)</td>
<td>5.7±0.4</td>
<td>6.3±0.8</td>
<td>0.005</td>
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Figure 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 E4 orf-1 gene expression post 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 mean ± SEM (n=3 per group). Values normalized to total PKB. *p<0.01.
C-D. Glucose uptake for insulin sensitive and insulin resistant donors. Donors were matched for BMI. Representative experiments shown as mean ± SEM.
C. Glucose uptake assay in adipose tissue from insulin sensitive donor 5 days post Ad-36 infection.
D. Glucose uptake assay in adipose tissue from insulin resistant (Type 2 diabetic) donor 5 days post Ad-36 infection. *p=0.036, **p=0.035.
**Figure 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 representative (n=3 per group). Mean ± SEM.

A-D. Time course gene expressions measured by qRT-PCR and normalized to cyclophilin

E-F. Number of cells per field. n=3 (explants) per group; 6 slices per explant were used for calculations. *p<0.05

G. Representative sections of adipose tissue used for calculation the number of cells in E and F. Sections from Ad-36 infected explants show a greater number of smaller cells on day 21 post inoculation. Red dots indicate cells counted as adipocytes.
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Figure 3. Increased adiponectin and FAS in Ad-36 infection of human adipose tissue. Experiments were performed as described in RESEARCH DESIGN AND METHODS.  
A-B. Time course gene expression post Ad-36 infection as measured by qRT-PCR and normalized to cyclophilin B. Values = MEAN ± SEM. Representative experiment, n=3 explants per group.  
C-D. Western blot analysis for adiponectin (media) and FAS protein levels.  
C. Upper panel. Autoradiograph of Western blot. Lower panel densitometry mean ± SEM (n=3 per group). Adiponectin protein levels were normalized to total protein content of individual explants.  
D. Upper panel shows autoradiograph. Lower panel densitometry mean ± SEM (n=3 per group). FAS protein levels were normalized to β-tubulin. *p<0.05, **p<0.01.
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Figure 4. No effect on adipogenic gene expression in Ad-2 infected human adipose tissue explants. Experiments were performed as described in RESEARCH DESIGN AND METHODS. All experiments representative (n=3 per group). Mean + SEM.

A-G. Time course gene expressions measured by qRT-PCR and normalized to cyclophilin.
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Figure 5. PI3K is required for Ad-36 induced improvement in adipose tissue metabolic profile. Experiments were performed as described in RESEARCH DESIGN AND METHODS. 

A) Inhibition of PI3K activity diminishes gene expression in human adipose tissue explants. Gene expressions were measured by qRT-PCR and normalized to cyclophilin B. Values expressed as arbitrary units. Representative experiment, n=3 per group. Mean ± SEM. PC-1, PECAM-1; Adiponectin WM = Wortmannin. *p<0.05, **p<0.01.

B) Time course of E4orf1 expression by Ad-36 infected explants with or without Wortmannin.

C-F) Ad-36 infection increases gene expression in human adipose tissue explants; effect is dependent on PI3K activity. Gene expressions were measured by qRT-PCR and normalized to Cyclophilin B. E4 = E4 orf-1, γ2 = PPARγ2, P1 = PECAM-1, M1 = MCP-1, W = Wortmannin. Values: Mean ± SEM (n=3 per group). Ad-36 (2.7 PFU/cell) group as compared to mock infection, *p<0.05. Ad-36 (2.7 PFU/cell) + Wortmannin group as compared to Ad-36 (2.7 PFU/cell) group, **p<0.05.

D-F) Time course of expression of selected adipogenic genes show greater expression in Ad-36 infected explants, which is attenuated by WM treatment.

G) Glucose uptake in human adipose tissue explants is increased by Ad-36 infection and dependent on PI3K activity. Insulin sensitive donor with a BMI of 22. Experiment representative with values = Mean ± SEM (n=3 per group). WM = Wortmannin. *p<0.05, mock infection as compared with mock infection + Wortmannin. **p<0.01. Ad-36 infection (2.7 PFU/cell) as compared to Ad-36 infection (2.7 PFU/cell) + Wortmannin. Glucose uptake assays performed 5 days post infections.
Ad-36 increases glucose uptake in human skeletal muscle cells
Ad-36 increases glucose uptake in human skeletal muscle cells

Figure 6. Effect of Ad-36 on cell signaling involved in glucose uptake. Experiments were performed as described in RESERCH DESIGN AND METHODS.
A. Western blot analysis shows that Ad-36 decreased insulin signaling proteins and their phosphorylations and increased Ras protein levels in adipose tissue explants. *p<0.05, Ad-36 infection (2.7 PFU/cell) as compared to Mock. **p<0.01.
B-E. Representative Western blot analysis of total protein extracts of hASC that were mock or Ad-36 infected in insulin stimulated and basal conditions. Experiments were conducted on three different patients. Densitometry mean ± SDM (n=3 per group)
B. Membranes were incubated with antibodies to IRS-1(pTyr 989), IRS-1, PKB (pSer473), PKB (pThr308), PKB or to β-Actin.
C. Densitometric analysis of IRS-1(pTyr 989) abundance.
D. Densitometric analysis of PKB (pSer473) abundance.
E. Densitometric analysis of PKB (pThr308) abundance.
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Figure 7: Effect of Ras siRNA transfection on glucose uptake in Ad-36 infected hASC.
Three days after Ad-36 infection (3.8 PFU/cell), hASC were transfected with 2 μg/ml Ras siRNA mixed with 2 fold SatisFection™ transfection reagent (Stratagene, La Jolla, CA). Three-days later, PI3k activation was determined by thin layer chromatography and glucose uptake was determined in absence and presence of insulin (100 nM insulin was added during the last 15 min of incubation). Data were mean ± SEM.

A. Ad-36 induced PI3K activation with or without Ras siRNA transfection.  
** P<0.01, ***P<0.001, Ad-36 vs control.  # P<0.01, Ad-36 vs Ad-36 + Ras siRNA.  

B. Ad-36 induced glucose uptake with or without Ras siRNA transfection.  
** P<0.01, ***P<0.01 Ad-36 vs control.  #P<0.05, ##P<0.01 Ad-36 vs Ad-36 + si Ras.  

C. hASC were mock infected or infected with Ad-36 or Ad-2 as described in Methods and transfected with null vector or a RAS specific shRNA vector. Compared to the “mock + null vector” group, “Ad-36 + Null vector” group but not “Ad-2 + null vector” had greater glucose uptake (p < .0001). Ras shRNA significantly altered the glucose uptake only in the Ad-36 infected group.  

D. Proteins harvested from samples of 7 C above, were used for Western blots. Representative blots from triplicate sample are presented.