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Insulin receptor-independent upregulation of cellular glucose uptake

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Abstract

Background—Cellular glucose uptake can be enhanced by upregulating Ras signaling in either insulin-dependent or - independent manner. In presence of insulin and intact insulin signaling, Ras has a negligible role in glucose uptake. Conversely, when insulin signaling is impaired in obesity or diabetes, the insulin-independent Ras pathway may be valuable for enhancing glucose disposal. We previously reported that Ad36, a human adenovirus, enhances cellular glucose uptake by upregulating the Ras/Glut4 pathway. Here, we investigated if Ad36-upregulated Ras via the insulin-independent pathway, to enhance glucose uptake. Furthermore, uncontrolled upregulation of Ras is linked with oncogenic cell transformation, if the tumor-suppressor gene p53 is also downregulated. Hence, we determined if upregulation of Ras by Ad36 would induce oncogenic cell transformation. Finally, we determined the relevance of Ad36 to insulin resistance in humans.

Methods—Insulin receptor (IR) was knocked down with small interfering RNA in 3T3-L1 adipocytes, to determine if Ad36 increases the Ras/Glut4 pathway and glucose uptake without IR-signaling. Next, the effects of Ad36 on cell transformation and p53 abundance were determined. Finally, overweight or obese women were screened for seropositivity to Ad36, as an indicator of natural Ad36 infection. Associations of Ad36 infection with adiposity and C-reactive proteins (CRPs)—two key markers of insulin resistance, and with glucose disposal, were determined.

Results—Unaffected by IR knock-down, Ad36 significantly increased the Ras pathway, Glut4 translocation and glucose uptake in 3T3-L1 adipocytes. Despite Ras upregulation, Ad36 did not transform 3T3-L1 cells. This may be because Ad36 significantly increased p53 protein in 3T3-L1

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cells or mice adipose tissue. Ad36 seropositivity was associated with greater adiposity and CRP levels, yet a significantly higher systemic glucose disposal rate.

Conclusions—Overall, the study offers Ras/Glut4 pathway as an alternate to enhance glucose disposal when insulin signaling is impaired, and, importantly, provides Ad36 as a tool to understand the modulation of that pathway.

Keywords

Ras; Ad36; PI3K; Glut 4; p53; IRS1

Introduction

Insulin signaling for glucose disposal could be broadly divided in to ‘proximal’ and ‘distal’ signaling. Proximal insulin signaling involves the binding of insulin to its receptor (IR) followed by the activation of IR substrates (IRS1 and IRS2). The distal insulin signaling includes the activation of phosphatidyl inositol-3 kinase (PI3K) by IRS1 and IRS2, leading to glucose transporter (Glut4)-mediated glucose disposal, via the activation of AKT2.¹ Most currently available anti-diabetic agents are insulin sensitizers or insulin secretagogues and therefore, require insulin signaling pathway for their action. However, some studies show that insulin-resistant states such as obesity or diabetes are often associated with a decrease in the binding of insulin to IR, and/or a decrease in insulin-stimulated activation of IRS,^{2–4} which may diminish the effectiveness of anti-diabetic agents dependent on this signaling. Therefore, next-generation agents that improve hyperglycemia without recruiting the proximal insulin signaling may be valuable in treating type 2 diabetes, the metabolic syndrome as well as type 1 diabetes. Accordingly, efforts are underway to enhance glucose disposal without recruiting insulin signaling^{5,6} or, by focusing on the distal insulin signaling pathway.^{7,8}

Ad36, a human adenovirus, increases cellular glucose uptake independent of insulin and improves glycemic control,^{9–11} and may offer a novel template to induce these effects without recruiting insulin signaling. In rodent models, experimental infection with Ad36 significantly improves systemic glycemic control in chow fed animals^{9,12} and even improves high-fat diet induced hyperglycemia and hepatic steatosis, without requiring a reduction in adiposity.⁹ In about 1500 human subjects, natural Ad36 infection predicted better glycemic control and lower hepatic lipid accumulation, independent of age, sex and adiposity.⁹ The congruence of animal and human data underscore the potential clinical relevance of these findings. Overall, Ad36 exhibits ‘insulin sparing action’,⁹ and offers a template to exogenously modulate glycemic control and hepatic steatosis, without reducing dietary fat intake or adiposity—a particularly appealing concept, given the challenges of weight loss or maintenance. Extensive cell signaling studies suggest adipose tissue is likely to have a key role in Ad36-induced improvements in glycemic control.^{9,10}

Ad36 robustly increases basal glucose uptake, which exceeds even the insulin-stimulated glucose uptake in the uninfected control cells.^{10,11} Like other human adenoviruses, Ad36 downregulates IR, IRS1 or IRS2 activation, yet, Ad36 recruits the distal insulin signaling

pathway (PI3K/AKT) via Ras activation, leading to greater Glut4 abundance and translocation and glucose disposal.^{9–11,13}

Ras is an important guanine tri phosphate-binding protein of cell survival, proliferation and growth,^{14,15} which has long been recognized to induce PI3K/AKT signaling pathway,¹⁶ adipocyte differentiation¹⁷ or mimic insulin action on glucose transporters.¹⁸ Ras-induced glucose disposal was ignored because it has a negligible role when insulin signaling is intact.¹⁹ Conversely, it may be very valuable, when insulin signaling is impaired. This was evident in a mouse model of transgenic overexpression of H-Ras in adipose tissue,²⁰ which showed better glycemic control and greater insulin-independent glucose uptake by adipocytes. Thus, Ras may be a valuable target to enhance cellular glucose uptake in obesity or diabetes, conditions when the insulin signaling is often impaired, and Ad36 appears to be an exogenous agent to effectively modulate this target. Therefore, we propose to revisit the role of Ras in insulin signaling-independent glucose uptake.

Binding of insulin to IR leads to (a) the activation of IRS proteins, and (b) the activation of Ras via Shc.²¹ Both of these key pathways in turn, activate the PI3K pathway.^{14,15} As Ad36 downregulates IR and IRS activation,^{10,11} we hypothesized that recruiting the Ras pathway allows Ad36 to activate PI3K pathway and increase glucose uptake, without IR signaling. We knocked down IR to test if without proximal insulin signaling, Ad36 can upregulate the Ras/ PI3K pathway and induce glucose uptake.

Conventionally, Ras is considered an oncogene. However, the oncogenic ability of Ras is expressed only in the absence or deficiency of p53—a key tumor-suppressor gene.²² Despite Ras- upregulation, Ad36 was not tumorigenic in animal studies lasting up to 7 months. Hence, we hypothesized and tested that Ad36 lacks the cell transformation ability, and which may be due to the upregulation of p53.

To determine the human relevance of the findings, and as a proof of concept, association of natural Ad36 infection with adiposity and indices of glycemic control were determined in overweight or obese women. Overall, this study determined a novel approach to exogenously enhance the Ras-mediated glucose uptake independent of IR signaling, and assessed its suitability for further developing the concept.

Materials and Methods

Experimental outlines are described below. Details of assays are presented under Techniques and assay section.

Experiment 1: glucose uptake in presence of IR knockdown

Basal glucose uptake—3T3-L1 mature adipocytes were transfected with small interfering RNA (siRNA) against IR, or with non-targeting (NT) siRNA, and 24-h post transfection, the cells were infected with five multiplicity of infection (MOI) Ad36, or mock infected to determine basal glucose uptake. This generated the following four groups of cells (NT, IR, NT+Ad36 and IR+Ad36).

Insulin-stimulated glucose uptake—Another set of 3T3-L1 mature adipocytes were transfected as above, with NT (two groups), or IR siRNA (two groups). One of the IR siRNA transfected groups was infected with five MOI of Ad36 and all other groups were mock infected. Two days post transfection all groups were exposed to insulin except one NT transfected group, before determining glucose uptake.

Each set of experiments was repeated at least twice and included at least six biological replicates each time. The representative data are presented.

Parallel experiments were set up to harvest whole cell lysates and cytoplasmic/membrane fraction proteins for western blot analyses as described in Techniques and assays section.

Experiment 2: cell transformation assay

3T3-L1 pre-adipocytes were infected with 10 MOI Ad36 or mock infected. Three days post infection, the two groups were plated for the soft agar assay to determine anchorage-independent colony formation—an indicator of cell transformation. A549 cells (human lung cancer cells; ATCC, Manassas, VA, USA, #CCL-185), were used as a positive control.

Experiment 3: p53 abundance

- (a) Mature 3T3-L1 adipocytes differentiated as described,²³ were infected with five MOI Ad36 or mock infected. Protein was extracted 24-h post infection to determine p53 expression by western blot assay.
- (b) p53 abundance was determined in mice adipose tissue obtained in an earlier experiment.⁹ These C57BL/6J mice were on 60% fat diet from week 8 of their age, and were infected with Ad36, Ad2 (a human adenovirus used as a control for infection) or mock infected when 14-week old. Retroperitoneal or epididymal adipose tissue obtained at 20-week post infection ($n = 3$ per group), were used to determine p53 abundance.

Experiment 4: association of Ad36 infection with glucose disposal in humans

Human baseline serum samples from two studies of non-diabetic over-weight and obese postmenopausal women ($N = 181$)^{24,25} who had a complete set of baseline data were screened *post hoc* for the presence of Ad36 neutralizing antibodies. The ‘constant virus-decreasing serum’ method was employed to determine the presence of neutralizing antibodies.²⁶ Both studies were approved by the University of Montreal ethics committee and all subjects gave written, informed consent.^{24,25} For these studies, the body fat mass and fat distribution (visceral and subcutaneous adipose tissue mass) were determined by dual X-ray absorptiometry and a General Electric High Speed Advantage CT scanner, respectively. Fasting insulin (radioimmunoassay), glucose (glucose oxidase) and plasma high sensitivity C-reactive protein (CRP) were assessed.²⁴ Insulin sensitivity was determined by measuring the glucose disposal rate during a hyperinsulinemic-euglycemic clamp.²⁴ Glucose disposal rates during the clamp were expressed as $\text{mg min}^{-1} \text{ kg}^{-1}$ fat-free mass or $\text{mg min}^{-1} \text{ kg}^{-1}$ body weight.

Techniques and assays

Differentiation of 3T3-L1 cells—Two days post confluence, 3T3-L1 preadipocytes maintained in Dulbecco's minimum essential medium (DMEM) containing 10% fetal bovine serum (FBS) were exposed to 20 ng ml⁻¹ insulin (Sigma Aldrich, St Louis, MO, USA, #I1882), 115 ng ml⁻¹ isobutylmethyl-xanthine (Sigma Aldrich, #I5879-1G) and 0.39 ng ml⁻¹ dexamethasone (Sigma Aldrich, #D4902). At 48 h after the induction, cells were switched to maintenance media of DMEM containing 10% FBS and 5 ng ml⁻¹ insulin.

siRNA transfection of 3T3-L1 adipocytes—The siRNA complex was formed in collagen-coated 48-well plates by adding equal volumes of the stock concentration of siRNA (INSR Dharmacon, Chicago, IL, USA, #L-04378-00-0005, NT Pool, #2 #D-001206-14-05) and DMEM and incubating at room temperature for 5 min.²⁷ Next, the transfection reagent DharmaFECT Duo (Dharmacon, #T-2010-2) was added to the complex (at 1.4 µl cm⁻²). The resuspended adipocytes were seeded at 1.165×10^5 per well in a total volume of 200 µl of DMEM into the siRNA complex and incubated at 37 °C, for 24 h. Next, the media were replaced with DMEM containing 10% FBS and antibiotics. Cells were mock infected or infected with Ad36 at five MOI for 24 h before determining the glucose uptake and harvesting for protein in radio-immunoprecipitation assay buffer and for RNA (TRI Reagent - Sigma Aldrich, #T9424) determinations.

Glucose uptake—Glucose uptake was conducted after overnight serum starvation as described.^{10,11} Briefly, cells in 24-well plates were serum starved for 2 h, and then washed 2 × with phosphate-buffered saline before adding 112.5 µl Krebs-Ringer phosphate buffer (136 mM NaCl, 4.7 mM KCl, 10 mM NaPO₄, 0.9 mM CaCl₂, 0.9 mM MgSO₄) with or without 100 nM bovine pancreas insulin (Sigma Aldrich, #15500) for 15 min. Two to three wells were treated with 100 nM cytochalasin B (Sigma Aldrich, #C6762) for subtraction of nonspecific glucose uptake. In all, 12.5 µl of 10 × isotope solution was then added to each well for a final concentration of 100 nM cold 2-deoxyglucose and 0.5 µCi ml⁻¹ [3 H]-2-deoxyglucose (PerkinElmer, Waltham, MA, USA, #NEC720A250UC) for 5 min. Immediately following the incubation, cells were washed in ice-cold phosphate-buffered saline. In total, 125 µl of 0.05% sodium dodecyl sulfate was then added to each well, and after incubating for 30 min at 37 °C, 450 µl of cell lysate was added to a scintillation vial by combining four wells and the remaining 25 µl was used for protein determination via bicinchoninic acid assay. The scintillation values were normalized to protein content of each well.

Soft agar assay—Minimum essential medium with 10% FBS and 0.6% agar was allowed to solidify at room temperature in six-well plates. Next, 0.5 ml of 80 000 cells ml⁻¹ suspended in minimum essential medium 10% FBS, 0.3% agar was added. After the agar set at room temperature, another 2 ml of minimum essential medium with 10% FBS, 0.6% agar was added as a top layer. Cells were re-fed with 0.5 ml of 2 × minimum essential medium with 20% FBS, every 7 days. Cells were fixed at days 7, 14 or 21 with 1 ml 0.01% crystal violet in 10% formalin overnight at 4 °C. Four 10 × images, one per plate quadrant, were counted for viable cells and colonies.

Western blotting—Cells were lysed in lysis buffer (20 mmol l⁻¹ Tris-HCl (pH 7.4), 50 mmol l⁻¹ NaCl, 1 mmol l⁻¹ EDTA, 1 mmol l⁻¹ EGTA, 1% Triton X-100, 25 mmol l⁻¹ NaF, 1 mmol l⁻¹ Na₃VO₄, 1 mmol l⁻¹ phenyl methyl sulfonyl fluoride, 5 µg ml⁻¹ leupeptin and 5 µg ml⁻¹ aprotinin) and lysates clarified by centrifuging at 10 000 g for 5 min at 4 °C. The protein content was determined by a bicinchoninic acid protein assay kit (Pierce Biotechnology, Rockford, IL, USA). Protein samples (30 µg) were resolved by 7.5 and 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membrane (0.45 µm pore size, Immobilon-p, Millipore, Temecula, CA, USA). The resolved protein samples were immunoblotted by incubating with primary antibodies toward Ras (Cell Signaling, Danvers, MA, USA, #3965), pAKT-Ser-473 (Cell Signaling, #9271), AKT (Cell Signaling, #4691), Glut4 (R&D, Minneapolis, MN, USA, #MAB1262), p53 (Cell Signaling, #2524), glyceraldehyde phosphate dehydrogenase (Cell Signaling, #2118S; Chemicon International, Billerica, MA, USA, #MAB374), followed by incubation with host-specific secondary antibodies. Immunoreactive bands were quantified by densitometric analysis using the AlphaEaseFC analyzer software (Miami, FL, USA).

Quantitative real-time qPCR—RNA from experimental cells was harvested with Tri-Reagent (MRC, Cincinnati, OH, USA, #TR118) and isolated using a combination of the Tri-Reagent and RNEasy Qiagen Kit (Qiagen, Valencia, CA, USA, #74104) as per the manufacturer's instructions. One µg of total RNA was reverse transcribed to complementary DNA using a cDNA Archive kit (ABI, Carlsbad, CA, USA, #4368813). In all, 30 ng of complementary DNA was loaded per well in duplicate for detection of IR using primer probe set INSR (Mm01211881_g1) from Applied Biosystems (Carlsbad, CA, USA). Gene expression was normalized to glyceraldehyde phosphate dehydrogenase (Applied Biosystems, ID# Mm99999915_g1). Stability of glyceraldehyde phosphate dehydrogenase across all treatment groups was confirmed by comparing its expression with 18-s mRNA expression from respective groups (data not shown). Taqman Universal PCR Mix was used according to the manufacturer's instructions (Applied Biosystems, #4304437). Complementary DNA was amplified on the ABI prism 7900HT using the standard curve method.

Cytoplasm – membrane fractionation—For detection of Glut4 translocation, cytoplasmic and membrane fractions from adipocytes and murine adipose tissue were isolated using a compartmental protein isolation kit (Millipore) as per the manufacturer's instructions. The protein concentration of the cytoplasmic and membrane protein fractions were estimated by bicinchoninic acid quantification method and Glut4 abundance was determined by western blot assay.

Statistical analysis

All data are expressed as mean ± s.d. All assays were performed with a minimum of three biological replicates. For all metabolic assays, 8 - 12 biological replicates were used, and normalized to protein content. Hypotheses were tested by comparing group means using a one-tailed Students *t*-test.

Human data

An independent *t*-test was performed to compare Ad36-positive and Ad36-negative individuals. Log-transformed values were used for analyses as indicated in Table 2. Indices of glycemic control were adjusted for body mass index. Statistical significance was considered at $P < 0.05$.

Results

Experiment 1

IR siRNA knocked down IR expression by 85%, compared with the groups with NT siRNA (Figure 1a). Compared with the mock-infected group (NT), Ad36-infected group (NT + Ad36) had about threefold greater basal glucose uptake (Figure 1b, $P = 0.0002$). Ad36 continued to increase basal glucose uptake despite IR knock down (KD) (Figure 1b, IR vs IR + Ad36, $P < 0.0001$). As expected, insulin significantly increased glucose uptake (Figure 1c, No Ins + NT vs Ins + NT, $P = 0.01$), which was abolished by IR KD (Figure 1c, Ins + NT vs Ins + IR, $P = 0.01$). However, addition of Ad36 increased insulin-stimulated glucose uptake by about threefold despite the IR KD (Figure 1c, Ins + IR vs Ins + IR + Ad36, $P = 0.004$). Thus, Ad36 robustly increases basal and insulin-stimulated glucose uptake and IR KD had no effect on it.

Ad36 upregulates Glut4 in a Ras-mediated, PI3K-dependent manner.^{10,11} In accordance, Ad36 significantly upregulated the Ras/PI3K pathway, as indicated by greater abundance of Ras, AKT phosphorylation and Glut 4 abundance and translocation (Figures 2a - d). Predictably, insulin stimulation increased Ras abundance in the mock-infected group (Figure 2a, NT vs Ins + NT, $P = 0.0002$) and the IR KD significantly reduced Ras abundance in the mock-infected group (INS + NT vs INS + IR), whereas, the addition of Ad36 rescued IR-induced KD of Ras (INS + IR vs INS + IR + Ad36) ($P = 0.0002$ for both comparisons). This indicated that insulin, but not Ad36, requires IR signaling to increase Ras abundance.

In mock-infected groups, insulin stimulation increased AKT- phosphorylation (p-AKT) and Glut4 abundance, which requires IR signaling, as indicated by a reduction in p-AKT and Glut4 abundance and translocation in IR KD groups (Figures 2b- d). In contrast, Ad36 enhanced p-AKT and Glut4 abundance, despite IR KD. Thus, the upregulation of Ras, p-AKT or Glut4 abundance, and glucose uptake is insulin signaling dependent in mock-infected groups, which Ad36 achieves without insulin stimulation or IR signaling (Figures 2a - d).

Experiment 2

As expected, A549 cells—a lung cancer cell line, formed colonies in soft agar (Figure 3), with 11.3 ± 9.1 (% mean $\pm$ s.d.) percentage of viable cells forming colonies 7 days post-plating. About 10% of viable A549 cells continued to form colonies at later time points. Although Ad36-infected 3T3-L1 cells did not form any colonies even up to 21 days post-plating (Table 1), indicating a non-transforming effect of Ad36 on these cells.

Experiment 3

p53 protein abundance was increased approximately fourfold in 3T3L1 adipocytes infected with Ad36 ($P<0.001$; Figure 4a). Similarly, compared with the mock-infected group, adipose tissue p53 abundance was increased ($P<0.05$) in high-fat-fed mice infected with Ad36, but in the Ad2-infected group (Figure 4b).

The upregulation in p53 may contribute to the lack of tumorigenic outcome in despite Ras upregulation by Ad36.

Experiment 4

Ten of 181 subjects (5.5%) were naturally infected with Ad36 as indicated by the presence of neutralizing antibodies. Despite similar age, the seropositive subjects had significantly greater body mass index (Table 2). Not the total fat %, but its distribution was different between two groups. Seropositive subjects had significantly greater peripheral and subcutaneous fat mass (determined by dual X-ray absorptiometry or computed tomography scan, respectively), without statistically significant differences in central visceral adipose tissue area. Interestingly, the seropositive subjects had significantly greater CRP levels, indicative of greater sub-clinical inflammation ($P=0.003$). Considering that CRP levels >10 mg l^{-1} are indicative of acute inflammation,²⁸ only those with CRP <10 mg dl^{-1} were analyzed ($N=164$ seronegatives, 6 seropositives).

CRP levels for all subjects were also higher for Ad36 seropositive subjects (4.8 ± 2.7 vs 3.1 ± 2.1 mg l^{-1}), at $P=0.07$. CRP levels are positively associated with adiposity²⁹ and insulin resistance.³⁰ Thus, the greater body mass index and CRP levels of seropositive subjects indicated insulin resistance. Yet, Ad36 seropositivity was associated with greater glucose disposal rate (Table 2). Fasting insulin or glucose levels did not differ significantly between the groups.

Discussion

Human adenoviruses

Adenoviruses are non-enveloped DNA viruses. In humans, adenoviruses commonly cause acute upper respiratory tract infections, enteritis or conjunctivitis³¹ and may also persist asymptomatically.³² Ad36 is serologically different compared with other 50 human adenoviruses.^{33,34} About 17% of the adults in the United States show natural Ad36 infection, as indicated by the presence of neutralizing antibodies to Ad36.³⁵ Experimental infection of Ad36 increases adiposity in several animal models, yet improves metabolic profile—including a relative hypolipidemia, lower inflammatory cytokine response and better glycemic control.^{9,12,36-40} Interestingly, natural Ad36 infection in humans is also associated with significantly better glycemic control.^{9,12} These data suggested that Ad36 may be capable of improving glycemic control in humans, and could be used as a tool to understand the signaling pathways to eventually improve glycemic control in humans.

Insulin signaling-independent effect

The potential of Ad36 to enhance glucose uptake independent of insulin offers a valuable opportunity. Interestingly, similar to the effect of other human adenoviruses,¹³ Ad36 decreases tyrosine phosphorylation of IR, IRS1 and IRS2 associated PI3K activities.^{9–11}

Yet, Ad36 robustly increases cellular basal glucose disposal,^{10,11} which is not further enhanced by insulin. These observations suggested that the proximal insulin signaling may not contribute to Ad36-induced upregulation of glucose uptake. By effectively knocking down the IR receptor, this study confirms that Ad36 promotes glucose uptake, even when the proximal insulin signaling is impaired. It appears that although Ad36 as well as other human adenoviruses impair IR signaling,¹³ the somewhat distinct potential of Ad36 to increase glucose disposal is due to its ability to upregulate distal insulin signaling via the PI3K pathway.^{10,11}

Besides the IR/IRS pathway, another approach to upregulate the PI3K pathway and the distal insulin signaling is via Ras.^{14,15} In fact, Ad36 requires Ras to upregulate the PI3K–Glut 4 pathway.^{10,11} Ras, however, could also be upregulated by insulin via the IR signaling.^{14,15} This is also evident in Figure 2a, which shows increase in Ras abundance by insulin, but, not when IR is knocked down. Thus, an unanswered question was if Ad36 upregulates Ras via IR signaling, or independent of it. This study confirms that Ad36 does not require IR signaling for the upregulation of Ras or the entire downstream PI3K/Glut4 pathway (Figure 2). Therefore, when insulin signaling is impaired/absent, Ras may offer an alternate approach to enhance glucose disposal, and Ad36 offers a tool to exogenously induce this pathway. Considering the known ability of Ras to increase glucose uptake independent of insulin signaling, exploiting its potentially valuable role in impaired insulin signaling should be revisited.

Ras and cell transformation

Uncontrolled activation of Ras pathway may have undesirable consequences such as oncogenic cell transformation.⁴¹ In addition, the upregulation of PI3K or IRS1 is also associated with cancer.^{42,43} Therefore, careful attention should be paid to agents that upregulate these pathways. Fortunately, just the upregulation of these pathways is not necessarily oncogenic. For instance, activation of Ras or PI3K cannot be tumorigenic without focal adhesion kinase,⁴⁴ a component of integrin signaling. Transgenic overexpression of Ras²⁰ was also not oncogenic. None of our animal experiments (over 10 experiments that lasted up to 7 months post infection) showed tumor formation in Ad36 group.^{9,12,36–38} Nonetheless, owing to the robust activation of Ras and PI3K, cell transforming effect of Ras activation by Ad36 was determined. No cell transformation was observed in Ad36- infected cells.

Furthermore, tumorigenesis is complex, but broadly described as a process that requires the activation of oncogenes and downregulation of tumor-suppressor genes.⁴⁵ Proto-oncogene Ras is incapable of transforming primary fibroblasts alone, particularly, in presence of intact p53 tumor-suppressor protein.²² Deficiency of p53 tumor-suppressor gene increases susceptibility to tumor formation,⁴⁶ whereas the restoration of p53 regresses tumors.⁴⁷ Ad36

strongly upregulates p53, which perhaps effectively counters any transformational influences of Ras.⁴⁸ Also, adiponectin, an insulin sensitizing adipocytokine that is strongly upregulated by Ad36 in the adipose tissue of mice used in experiment 3 (ref.⁹) and in fat cells,¹⁰ is reported to exert its anti-carcinogenic effect via the upregulation of p53.⁴⁹ Therefore, the upregulation of p53 by Ad36 seems to indicate an anti- tumorigenic instead of a tumorigenic profile. Studies to knock down p53 in presence of Ad36, are required to confirm the role of p53 in blocking cell transformation.

In summary, the upregulation of Ras by Ad36 is not tumorigenic in animal models we studied, and the adipose tissue and adipocytes do not exhibit a tumorigenic profile in association with Ras upregulation. Although additional and careful studies are needed, these observations collectively support the non-oncogenicity of Ras overexpression observed in mice.²⁰

Ras and glucose disposal

Thus, oncogenic transformation is not an inevitable outcome of Ras upregulation. Ras has an important role in cell physiology. The role of Ras in glucose disposal generated considerable interest in the 1990s, but, perhaps due to the emergence of the thiazolidinediones class of anti-diabetic drugs, the interest in Ras as a potential anti-diabetic drug target seems to have faded. Now, with the use of at least some thiazolidinediones likely to eclipse because of recently reported adverse effects,⁵⁰ we would like draw attention to revisit Ras signaling for innovatively enhancing the glucose disposal. Ad36 appears to provide an ideal tool to study this signaling. Ad36 bypasses the proximal insulin signaling—the usual inducer of Ras, yet recruits Ras to increase glucose uptake, and increases p53—which likely blocks any undesirable effects of Ras upregulation.

Ad36 and glucose disposal in humans

Association of Ad36 with better glycemic control in humans is previously reported.⁹ In addition, the human data here suggest that Ad36 infection improves glucose disposal even in a population with two key markers associated with insulin resistance—greater body mass index and CRP levels. Peripheral (vs visceral) adiposity is associated with insulin sensitivity,⁵¹ which agrees well with greater subcutaneous adiposity observed in Ad36-infected subjects. Moreover, the findings are also consistent with animal data, which show greater adiposity and markers of inflammation, yet better glycemic control in response to Ad36 infection.^{9,12,39} Although these data reveal an association between Ad36 infection and glucose disposal, body fat distribution and CRP levels and serve as an important proof of concept, the preliminary nature of this cross-sectional study of limited sample size should be noted. Longer-term follow-up data in a larger sample of men and women of different races and geographical locations is required to further test these assertions.

Further research is required to identify and characterize the candidate Ad36 protein that enhances glucose disposal and to carefully determine the precise host signaling it modulates. Although, the safety of the induction of Ras pathway should be carefully studied further, these data raise the possibility of reconsidering Ras as a target when insulin signaling is impaired or absent. In particular, the study (a) offers Ras/PI3K pathway as an alternate to

bypass the proximal insulin signaling for enhancing glucose disposal, and (b) provides Ad36 as a tool to exogenously modulate the Ras/PI3K pathway independent of IR signaling or insulin resistance. Collectively, the findings may offer a template to develop novel agents for the treatment of hyperglycemia associated with obesity, and type 1 or type 2 diabetes.

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References

1. Jiang ZY, Zhou QL, Coleman KA, Chouinard M, Boese Q, Czech MP. Insulin signaling through Akt/protein kinase B analyzed by small interfering RNA-mediated gene silencing. *Proc Natl Acad Sci USA*. 2003; 100:7569–7574. [PubMed: 12808134]
2. Goodyear LJ, Giorgino F, Sherman LA, Carey J, Smith RJ, Dohm GL. Insulin receptor phosphorylation, insulin receptor substrate-1 phosphorylation, and phosphatidylinositol 3-kinase activity are decreased in intact skeletal muscle strips from obese subjects. *J Clin Invest*. 1995; 95:2195–2204. [PubMed: 7537758]
3. Pessin JE, Saltiel AR. Signaling pathways in insulin action: molecular targets of insulin resistance. *J Clin Invest*. 2000; 106:165–169. [PubMed: 10903329]
4. Sesti G, Federici M, Hribal ML, Lauro D, Sbraccia P, Lauro R. Defects of the insulin receptor substrate (IRS) system in human metabolic disorders. *FASEB J*. 2001; 15:2099–2111. [PubMed: 11641236]
5. Klip A. The many ways to regulate glucose transporter 4. *Appl Physiol Nutr Metab*. 2009; 34:481–487. [PubMed: 19448718]
6. Tanaka T, Nakatani K, Morioka K, Urakawa H, Maruyama N, Kitagawa N, et al. Nitric oxide stimulates glucose transport through insulin-independent GLUT4 translocation in 3T3-L1 adipocytes. *Eur J Endocrinol*. 2003; 149:61–67. [PubMed: 12824867]
7. Perdomo G, Martinez-Brocca MA, Bhatt BA, Brown NF, O'Doherty RM, Garcia-Ocana A. Hepatocyte growth factor is a novel stimulator of glucose uptake and metabolism in skeletal muscle cells. *J Biol Chem*. 2008; 283:13700–13706. [PubMed: 18362143]
8. Frevert EU, Kahn BB. Differential effects of constitutively active phosphatidylinositol 3-kinase on glucose transport, glycogen synthase activity, and DNA synthesis in 3T3-L1 adipocytes. *Mol Cell Biol*. 1997; 17:190–198. [PubMed: 8972199]
9. Krishnapuram R, Dhurandhar EJ, Dubuisson O, Kirk-Ballard H, Bajpeyi S, Butte N, et al. Template to improve glycemic control without reducing adiposity or dietary fat. *Am J Physiol Endocrinol Metab*. 2011; 300:E779–E789. [PubMed: 21266671]
10. Rogers PM, Mashtalir N, Rathod MA, Dubuisson O, Wang ZQ, Dasuri K, et al. Metabolically favorable remodeling of human adipose tissue by human adenovirus Ad-36. *Diabetes*. 2008; 57:2321–2331. [PubMed: 18599527]
11. Wang ZQ, Cefalu WT, Zhang XH, Yongmei Y, Qin J, Son L, et al. Human adenovirus type 36 enhances glucose uptake in diabetic and non-diabetic human skeletal muscle cells independent of insulin signaling. *Diabetes*. 2008; 57:1805–1813. [PubMed: 18420488]
12. Pasarica M, Shin AC, Yu M, Ou Yang HM, Rathod M, Jen KL, et al. Human adenovirus 36 induces adiposity, increases insulin sensitivity, and alters hypothalamic monoamines in rats. *Obesity (Silver Spring)*. 2006; 14:1905–1913. [PubMed: 17135605]

13. Jiang S, Gavrikova TA, Pereboev A, Messina JL. Adenovirus infection results in alterations of insulin signaling and glucose homeostasis. *Am J Physiol Endocrinol Metab.* 2010; 298:E1295–E1304. [PubMed: 20388825]
14. Shaw RJ, Cantley LC. Ras, PI(3)K and mTOR signaling controls tumour cell growth. *Nature.* 2006; 441:424–430. [PubMed: 16724053]
15. Suire S, Condcliffe AM, Ferguson GJ, Ellson CD, Guillou H, Davidson K, et al. Gbetagammias and the Ras binding domain of p110gamma are both important regulators of PI(3)Kgamma signaling in neutrophils. *Nat Cell Biol.* 2006; 8:1303–1309. [PubMed: 17041586]
16. Sasaki AT, Chun C, Takeda K, Firtel RA. Localized Ras signaling at the leading edge regulates PI3K, cell polarity, and directional cell movement. *J Cell Biol.* 2004; 167:505–518. [PubMed: 15534002]
17. Benito M, Porras A, Nebreda AR, Santos E. Differentiation of 3T3-L1 fibroblasts to adipocytes induced by transfection of ras oncogenes. *Science.* 1991; 253:565–568. [PubMed: 1857988]
18. Kozma L, Baltensperger K, Klarlund J, Porras A, Santos E, Czech MP. The ras signaling pathway mimics insulin action on glucose transporter translocation. *Proc Natl Acad Sci USA.* 1993; 90:4460–4464. [PubMed: 8389451]
19. Dorrestijn J, Ouwens DM, Van den Berghe N, Bos JL, Maassen JA. Expression of a dominant-negative Ras mutant does not affect stimulation of glucose uptake and glycogen synthesis by insulin. *Diabetologia.* 1996; 39:558–563. [PubMed: 8739915]
20. Houseknecht KL, Zhu AX, Gnudi L, Hamann A, Zierath JR, Tozzo E, et al. Overexpression of Ha-ras selectively in adipose tissue of transgenic mice. Evidence for enhanced sensitivity to insulin *J Biol Chem.* 1996; 271:11347–11355. [PubMed: 8626688]
21. Ceresa BP, Pessin JE. Insulin regulation of the Ras activation/inactivation cycle. *Mol Cell Biochem.* 1998; 182:23–29. [PubMed: 9609111]
22. Finlay CA, Hinds PW, Levine AJ. The p53 proto-oncogene can act as a suppressor of transformation. *Cell.* 1989; 57:1083–1093. [PubMed: 2525423]
23. Rathod M, Rogers PM, Vangipuram SD, McAllister EJ, Dhurandhar NV. Adipogenic cascade can be induced without adipogenic media by a human adenovirus. *Obesity.* 2009; 17:657–664. [PubMed: 19165154]
24. Brochu M, Malita MF, Messier V, Doucet E, Strychar I, Lavoie JM, et al. Resistance training does not contribute to improving the metabolic profile after a 6-month weight loss program in overweight and obese postmenopausal women. *J Clin Endocrinol Metab.* 2009; 94:3226–3233. [PubMed: 19567540]
25. Lavoie ME, Rabasa-Lhoret R, Ziai S, Lavoie JC. Blood glutathione peroxidase activity in relation with the risk of cardiovascular diseases. *J Diabetes Metab.* 2011; 2:2–6.
26. Atkinson RL, Dhurandhar NV, Allison DB, Bowen RL, Israel BA, Albu JB, et al. Human adenovirus-36 is associated with increased body weight and paradoxical reduction of serum lipids. *Int J Obes (Lond).* 2005; 29:281–286. [PubMed: 15611785]
27. Kilroy G, Burk DH, Floyd ZE. High efficiency lipid-based siRNA transfection of adipocytes in suspension. *PLoS One.* 2009; 4:e6940. [PubMed: 19759827]
28. Jialal I, Devaraj S, Venugopal SK. C-reactive protein: risk marker or mediator in atherothrombosis? *Hypertension.* 2004; 44:6–11. [PubMed: 15148294]
29. Visser M, Bouter LM, McQuillan GM, Wener MH, Harris TB. Elevated C-reactive protein levels in overweight and obese adults. *JAMA.* 1999; 282:2131–2135. [PubMed: 10591334]
30. Bluher M, Fasshauer M, Tonjes A, Kratzsch J, Schon MR, Paschke R. Association of interleukin-6, C-reactive protein, interleukin-10 and adiponectin plasma concentrations with measures of obesity, insulin sensitivity and glucose metabolism. *Exp Clin Endocrinol Diabetes.* 2005; 113:534–537. [PubMed: 16235156]
31. Foy, HM.; Grayston, JT. Adenoviruses. In: Evans Alfred, S., editor. *Viral Infections of Humans: Epidemiology and Control.* Plenum Medical; New York: 1976. p. 53-70.
32. Horvath J, Palkonyay L, Weber J. Group C adenovirus DNA sequences in human lymphoid cells. *J Virol.* 1986; 59:189–192. [PubMed: 3486983]

33. Hierholzer JC, Wigand R, Anderson LJ, Adrian T, Gold JW. Adenoviruses from patients with AIDS: a plethora of serotypes and a description of five new serotypes of subgenus D (types 43-47). *J Infect Dis.* 1988; 158:804–813. [PubMed: 2844918]
34. Wigand R, Gelderblom H, Wadell G. New human adenovirus (candidate adenovirus 36), a novel member of subgroup D. *Arch Virol.* 1980; 64:225–233. [PubMed: 7406681]
35. Atkinson R, Dhurandhar N, Allison D, Bower R, Israel B. Evidence for an association of an obesity virus with human obesity at three sites in the United States. *Int J Obes.* 1998; 22:S57.
36. Dhurandhar NV, Israel BA, Kolesar JM, Mayhew GF, Cook ME, Atkinson RL. Increased adiposity in animals due to a human virus. *Int J Obes Relat Metab Disord.* 2000; 24:989–996. [PubMed: 10951537]
37. Dhurandhar NV, Israel BA, Kolesar JM, Mayhew G, Cook ME, Atkinson RL. Transmissibility of adenovirus-induced adiposity in a chicken model. *Int J Obes Relat Metab Disord.* 2001; 25:990–996. [PubMed: 11443497]
38. Dhurandhar NV, Whigham LD, Abbott DH, Schultz-Darken NJ, Israel BA, Bradley SM, et al. Human adenovirus Ad-36 promotes weight gain in male rhesus and marmoset monkeys. *J Nutr.* 2002; 132:3155–3160. [PubMed: 12368411]
39. Pasarica M, Loiler S, Dhurandhar NV. Acute effect of infection by adipogenic human adenovirus Ad36. *Arch Virol.* 2008; 153:2097–2102. [PubMed: 18830560]
40. Dhurandhar NV. A framework for identification of infections that contribute to human obesity. *Lancet Infect Dis.* 2011; 11:963–969. [PubMed: 22115071]
41. Bos JL. Ras oncogenes in human cancer: a review. *Cancer Res.* 1989; 49:4682–4689. [PubMed: 2547513]
42. Slattery ML, Samowitz W, Curtin K, Ma KN, Hoffman M, Caan B, et al. Associations among IRS1, IRS2, IGF1, and IGF1R genetic polymorphisms and colorectal cancer. *Cancer Epidemiol Biomarkers Prev.* 2004; 13:1206–1214. [PubMed: 15247132]
43. Bader AG, Kang S, Zhao L, Vogt PK. Oncogenic PI3K deregulates transcription and translation. *Nat Rev Cancer.* 2005; 5:921–929. [PubMed: 16341083]
44. Pylayeva Y, Gillen KM, Gerald W, Beggs HE, Reichardt LF, Giancotti FG. Ras- and PI3K-dependent breast tumorigenesis in mice and humans requires focal adhesion kinase signaling. *J Clin Invest.* 2009; 119:252–266. [PubMed: 19147981]
45. Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell.* 2000; 100:57–70. [PubMed: 10647931]
46. Donehower LA, Harvey M, Slagle BL, McArthur MJ, Montgomery CA Jr, Butel JS, et al. Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. *Nature.* 1992; 356:215–221. [PubMed: 1552940]
47. Ventura A, Kirsch DG, McLaughlin ME, Tuveson DA, Grimm J, Lintault L, et al. Restoration of p53 function leads to tumour regression *in vivo*. *Nature.* 2007; 445:661–665. [PubMed: 17251932]
48. Boiko AD, Porteous S, Razorenova OV, Krivokrysenko VI, Williams BR, Gudkov AV. A systematic search for downstream mediators of tumor suppressor function of p53 reveals a major role of BTG2 in suppression of Ras-induced transformation. *Genes Dev.* 2006; 20:236–252. [PubMed: 16418486]
49. Mistry T, Digby JE, Desai KM, Randeva HS. Leptin and adiponectin interact in the regulation of prostate cancer cell growth via modulation of p53 and bcl-2 expression. *BJU Int.* 2008; 101:1317–1322. [PubMed: 18279445]
50. Lipscombe LL, Gomes T, Levesque LE, Hux JE, Juurlink DN, Alter DA. Thiazolidinediones and cardiovascular outcomes in older patients with diabetes. *JAMA.* 2007; 298:2634–2643. [PubMed: 18073359]
51. McLaughlin T, Lamendola C, Liu A, Abbasi F. Preferential fat deposition in subcutaneous versus visceral depots is associated with insulin sensitivity. *J Clin Endocrinol Metab.* 2011; 96:E1756–E1760. [PubMed: 21865361]

Figure 1.

Ad36 increases glucose uptake independent of IR signaling. (a) IR knock down by siRNA was determined by quantitative reverse transcriptase -PCR (bar graph) and western blotting (top panel). 2-Deoxyglucose uptake under basal (b) and insulin-stimulated (c) conditions 2 days post transfection was determined in 3T3-L1 cells. NT, cells transfected with NT siRNA; IR, cells transfected with IR siRNA; NT + Ad36, cells transfected with NT siRNA and infected with Ad36; IR + 36, cells transfected with IR siRNA and infected with Ad36; No Ins + NT, cells transfected with NT siRNA, no insulin; Ins + NT, cells transfected with NT siRNA + insulin; Ins + IR, cells transfected with IR siRNA + insulin; Ins + IR + Ad36, cells transfected with IR siRNA and infected with Ad36 + insulin.

Figure 2.

Ad36 upregulates Ras/PI3K/Glut4 pathway independent of IR signaling. Abundance of (a) Ras, (b) p-AKT and (c) Glut4 proteins was determined by western blot analyses (* $P < 0.01$ or better). The same blots were stripped and re-probed with anti- glyceraldehyde phosphate dehydrogenase (GAPDH) antibody to normalize for protein loading. After densitometry, integrated density value (IDV) for each protein band was determined and normalized levels of Ras and Glut4 were calculated by dividing the IDV of a protein band by the IDV of the GAPDH. The cytoplasmic and membrane fractions in (d) were first normalized to their respective housekeeping genes and then a ratio of membrane to cytoplasm calculated which is expressed on the y axis.

Figure 3.

Anchorage-independent colony formation – cell transformation assay. Cell formation determined by anchorage-independent colony formation in 3T3-L1 pre-adipocytes mock infected or infected with Ad36. A549, human lung cancer cells were used as positive control. Inset shows colony formation in the positive control cells, which are absent in Ad36 or mock-infected cells.

Figure 4.

Ad36 upregulates p53 protein abundance. Cellular p53 expression was determined in adipose tissue and 3T3L1 adipocytes by western blot analysis. p53 levels were detected by immunoblot analysis utilizing mouse monoclonal anti-p53 antibody. The same blot was stripped and re-probed with anti-glyceraldehyde phosphate dehydrogenase (GAPDH) antibody to normalize for protein loading. After densitometry, integrated density value (IDV) for each protein band was determined and normalized levels of p53 calculated by

dividing the IDV of a protein band by the IDV of the GAPDH within the same sample. (a) p53 expression in 3T3-L1 adipocytes. (b) p53 expression in adipose tissue of HF fed mice. * $P < 0.05$ and ** $P = 0.001$ compared with respective mock groups.

Table 1
Percentage of viable cells that formed colonies (% $\pm$ s.d.)

	<i>Day 7</i>	<i>Day 14</i>	<i>Day 21</i>
A549 cells	11.3 $\pm$ 9.1	9.2 $\pm$ 2.1	9.9 $\pm$ 8.6
3T3-L1 cells/Ad36 infected	0	0	0
3T3-L1 cells/mock infected	0	0	0

Table 2
Comparison of seropositive and seronegative women

<i>Variables</i>	<i>Ad36 negative (n= 171)</i>	<i>Ad36 positive (n = 10)</i>	<i>P-value</i>
Age (years)	57.3 ± 4.7	56.4 ± 3.0	0.539
BMI (kg m ⁻²)	32.9 ± 4.4	36.3 ± 4.4	0.017
Fat-free mass (kg)	45.6 ± 6.6	47.4 ± 4.4	0.445
Fat mass (%)	46.4 ± 4.5	48.6 ± 3.1	0.152
Peripheral fat mass (kg)	19.7 ± 4.8	23.0 ± 5.1	0.039
Central fat mass (kg)	19.1 ± 5.1	20.7 ± 3.4	0.312
Visceral adipose tissue (cm ²)	189.6 ± 54.7	204.4 ± 62.5	0.434
Subcutaneous adipose tissue (cm ²)	481.5 ± 116.6	570.0 ± 108.4	0.028
hs-CRP (mg l ⁻¹) ^{a,b}	3.6 ± 3.6	7.8 ± 5.2	0.003
Fasting glucose (mmol l ⁻¹) ^c	5.2 ± 0.5	5.2 ± 0.5	0.836
Fasting insulin (μU ml ⁻¹) ^{a,c}	15.3 ± 6.6	15.7 ± 6.1	0.668
Glucose disposal rate (mg min ⁻¹ kg ⁻¹ BW) ^c	6.1 ± 1.7	6.7 ± 2.5	0.044
Glucose disposal rate (mg min ⁻¹ kg ⁻¹ FFM) ^c	11.5 ± 3.2	13.0 ± 4.7	0.068

Abbreviations: BMI, body mass index; BW, body weight; FFM, fat free mass; hs-CRP, high sensitivity C-reactive protein.

^aLog-transformed values were used for analyses.

^bSubjects with CRP <10mg l⁻¹.

^cAdjusted for BMI. Bold entries denote statistically significantly different variables.