Title

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Journal

International Journal of Obesity, 31(1)

ISSN

0307-0565

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Publication Date

2007

DOI

10.1038/sj.ijo.0803366

Peer reviewed

International Journal of Obesity (2006) 1–10

0307-0565/06 $30.00
www.nature.com/ijo

ORIGINAL ARTICLE

Adipogenic human adenovirus-36 reduces leptin expression and secretion and increases glucose uptake by fat cells

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Objective: Human adenovirus Ad-36 causes adiposity in animal models and enhances differentiation and lipid accumulation in human and 3T3-L1 preadipocytes, which may, in part, explain the adipogenic effect of Ad-36. We determined the consequences of Ad-36 infection on leptin and glucose metabolism in fat cells.
Design: 3T3-L1 preadipocytes were used to determine the effect of infection by human adenoviruses Ad-36, Ad-2, Ad-9 and Ad-37 on leptin secretion and lipid accumulation. Rat primary adipocytes were used to determine the effect of Ad-36 infection on leptin secretion and glucose uptake *in vitro*. Furthermore, the effect of Ad-36 on expressions of leptin and selected genes of *de novo* lipogenesis pathway of visceral adipose tissue were compared *ex vivo*, between Ad-36 infected and uninfected control rats.
Results: Ad-36 suppressed the expression of leptin mRNA in 3T3-L1 cells by approximately 58 and 52% on days 3 and 5 post-infection, respectively. Leptin release normalized to cellular lipid content was 51% lower ($P<0.002$) in the Ad-36 infected 3T3-L1 cells. Lipid accumulation was significantly greater and leptin secretion was lower for the 3T3-L1 cells infected with other human adenoviruses Ad-9, Ad-36, or Ad-37. Whereas, human adenovirus Ad-2 did not influence cellular lipid accumulation or the leptin release. In rat primary adipocytes, Ad-36 reduced leptin release by about 40% in presence of 0.48 ($P<0.01$) or 1.6 nM insulin ($P<0.05$) and increased glucose uptake by 93% ($P<0.001$) or 18% ($P<0.05$) in presence of 0 or 0.48 nM insulin, respectively. Next, the adipose tissue of Ad-36 infected rats showed two to fivefold lower leptin mRNA expression, and 1.6- to 21-fold greater expressions for acetyl Co-A carboxylase-1 and 1.2- to 6.3-fold greater expressions for fatty acid synthase, key genes of *de novo* lipogenesis, compared to the uninfected weight and adiposity matched controls.
Conclusion: The *in vitro* and *ex vivo* studies show that Ad-36 modulates adipocyte differentiation, leptin production and glucose metabolism. Whether such a modulation contributes to enhanced adipogenesis and consequent adiposity in Ad-36 infected animals or humans needs to be determined.
International Journal of Obesity advance online publication, 16 May 2006; doi:10.1038/sj.ijo.0803366

Keywords: infectobesity; adiposity; Ad-36; glucose; lactate; 3T3-L1

Introduction

The etiology of obesity is multifactorial and understanding the contribution of various causative factors is critical for its successful management. In addition to genetic, behavioral, endocrinal and other factors, viral infections have been proposed as one of the nine causative factors for obesity[1] and are recently receiving increasing attention.[2,3] To date, seven pathogens have been reported to promote adiposity in animal models.[4,5] Adenovirus type 36 (Ad-36) is the first human virus reported to induce adiposity along with a paradoxical relative hypolipidemia in experimentally infected chickens, mice and non-human primates.[6–8] Ad-36 antibodies are associated with obesity and reduced cholesterol levels in humans.[9] The prevalence of Ad-36 antibodies was 30% in the obese compared with 11% in the non-obese subjects screened, and the antibody positive subjects had greater body weights but lower-serum lipids, findings similar to those observed in the animal models.[10]

The absence of overt hypothalamic lesions and a positive correlation of Ad-36 DNA quantity in adipose tissue with the mass of that adipose depot in Ad-36 infected animals

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Received 22 December 2005; revised 23 March 2006; accepted 24 March 2006

(unpublished observations, Dhurandhar *et al*.) suggested a direct effect of the virus on adipose tissue, as opposed to a centrally mediated mechanism of action. We recently reported that Ad-36 enhances differentiation of 3T3-L1 as well as human preadipocytes,[11] which may, in part, explain the *in vivo* adipogenic effect of Ad-36. Ad-36 infection increased the number of adipocytes, lipid content and activity of glycerol 3-phosphate dehydrogenase (G-3PDH), a differentiation specific enzyme marker of adipocytes.[11]

Subsequent to observing the primary effect of Ad-36 on preadipocyte differentiation process, we proceeded to investigate functional modulations in the infected adipocytes. Production and release of adipocytokines is one of the important functions of adipocytes. Leptin, a protein involved in body weight regulation, is one of the most extensively studied adipocytokines.[12,13] During adipocyte differentiation, leptin expression increases concomitantly with lipid droplet accumulation.[14] Leptin appears to have paracrine feedback effects on adipocyte metabolism.[15,16] Leptin stimulates lipolysis, inhibits the expression of genes of lipid synthesis such as fatty acid synthase (FAS) and upregulates adipocyte genes involved in lipid oxidation.[17,18] Adenovirus-vector induced hyperleptinemia in rats depletes body fat.[19] Conversely, adipogenic agents like the thiazolinediones and valproic acid reduce leptin secretion.[20–22] Furthermore, adipocyte-selective reduction of leptin receptors leads to adiposity in mice, despite a normal level of hypothalamic leptin receptors and normal food intake.[16] These findings suggest adipogenic role of hypoleptinemia. Therefore, we hypothesized that a relative hypoleptinemia produced locally in adipose tissue may act via autocrine/paracrine pathway and contribute to enhanced preadipocyte differentiation, lipid accumulation and fatty acid synthesis in cells infected with Ad-36.[11] Moreover, leptin impairs insulin sensitivity and glucose uptake in adipocytes.[23,24] Therefore, we further hypothesized that Ad-36-induced relative hypoleptinemia will be accompanied by an increase in glucose uptake in adipocytes.

As described below, we investigated the effect of Ad-36 infection on expression and secretion of leptin by 3T3-L1 cells and screened other human adenoviruses for similar effects. Next, we determined the effect of Ad-36 on leptin secretion and glucose uptake in rat primary adipocytes. Considering the likely conversion of glucose in adipocytes to lipids, we determined the effect on expressions of leptin and genes of *de novo* lipogenesis in the adipose tissue of Ad-36 infected rats.

Research methods and procedures

Materials

Media: minimum essential media Eagle (MEM) (Cat # M-0643, Sigma Chemicals, St Louis, MO, USA) with non-essential amino acids, Earle's salts and L-glutamine and 10% fetal bovine serum (FBS, GIBCO, Carlsbad, CA, USA) was used for growing A549 cells and Dulbecco's modified Eagle's Media (DMEM) (Cat # M-0643, Sigma Chemicals) with 10% fetal bovine serum (FBS) was used to grow 3T3-L1 cells.

Tissue culture: A549 cells (human bronchial carcinoma cells) (ATCC Cat # CCL-185) were used for harvesting adenoviruses and 3T3-L1 cells (ATCC, Cat # CCL-92-1) were used for the differentiation experiments.

Virus: Ad-36 obtained from ATCC (Cat # VR-913) and plaque purified, was used for the experiments. The titer of virus stocks was determined as described previously.[6]

Experiments

Experiment 1. Leptin gene expression:. (a) Ad-36 inoculation and differentiation of 3T3-L1 cells: 3T3-L1 cells were grown in six-well (35 mm) plates and cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic–antimycotic (Penicillin-Streptomycin and Amphotericin B). At confluence (day 0), the media was removed from each well and the cells were inoculated with Ad-36 (Ad-36 group) (multiplicity of infection, MOI 3.8) or media (Control group) and incubated for 1 h at 37°C Next, this inoculum was replaced with 3 ml of differentiation media (MDI, containing DMEM, and 10% bovine serum with 1 × antibiotic–antimycotic, 10 μg/ml insulin, 39 μg/ml dexamethasone and 115 μg/ml 3-isobutyl-1-methylxanthine). After incubation for 48 h at 37°C (day 2), the differentiation media was replaced with 10% FBS-DMEM containing 0.001% insulin (maintenance media). Maintenance media was replaced every 2 days.

(b) RNA extraction: RNA was extracted from control and Ad-36 inoculated 3T3-L1 cells on days 0, 1, 2, 3 and 5 post-inoculation using TRIZOL reagent (Sigma cat # T-9424) followed by chloroform treatment and RNA precipitation by isopropyl alcohol. Residual genomic DNA was removed using RNase-free DNase (Ambion, Austin, TX, USA). Sample RNA concentrations and purity were determined by spectrophotometry. The integrity of all RNA samples was confirmed by electrophoresis in denaturing formaldehyde-containing gels. This RNA was used to determine leptin mRNA level by qualitative RT-PCR and real-time PCR assays as described below (c and d). Separate experiments were conducted to provide samples for the qualitative and quantitative RT-PCR assays.

(c) Leptin gene expression by qualitative RT-PCR: The following primer pairs were designed to detect leptin mRNA by RT-PCR using one step RT-PCR kit (Clontech RT-PCR KIT, Cat # K1403-2).

Forward primer: 5′-**GGCTTTGGTCCTATCTGTCTTATGTTC**-3′
Reverse primer: 5′-**CCTGTTGATAGACTGCCAGAGTCTG**-3′

Six replicate samples were used for the Control and Ad-36 groups. RNA (1 μg) sample was used for each reaction. Linearity range of PCR cycle numbers was decided by

linearity test. The following time and temperature conditions were used for the assay: Reverse transcription: 1 h, 50°C PCR: denaturation 30 s at 94°C annealing 30 s at 65°C extension 1 min at 68°C and final extension 2 min at 68°C Inactivated reverse transcriptase (by heating for 5 min at 95°C) was used as a negative control for every sample and 18 s rRNA expression was used as a positive control for the assay. Leptin gene expression was quantified using Bio-Rad Chemi-imager (Cat # 170-8060) and normalized to 18sRNA expression.

(d) Leptin gene expression by real-time RT-PCR: Of total RNA 1 μg was reverse-transcribed to cDNA by using iScript^{TM} cDNA Synthesis Kit (Cat # 170-8890, Bio-Rad, Hercules, CA, USA) per the manufacturer's protocol. Three replicate samples (obtained from experiments repeated thrice) were used for the control and Ad-36 groups. The 20 μl reactions were incubated at 25°C for 5 min, then at 42°C for 30 min followed by 85°C for 5 min, to inactivate the reverse transcriptase. The amount of leptin mRNA relative to the β-actin endogenous control was determined using real-time, quantitative PCR. The sequence of the leptin primers was 5′-GAATGCTGAAGTTTCAAAGG-3′, forward primer and 5′-GGAGAGAAATGAATGATGGA-3′ reverse primer of the Leptin gene cDNA sequence. The expected product sizes for the β-actin and leptin primers were 143 and 121 bp, respectively. Real-time quantitative PCR was performed in the iCycler iQ System (Bio-Rad) using SYBER Green detection system (Cat # 170-8880, Bio-Rad).

Briefly, to 2.5 μl of the 10 × SYBR green buffer, 1 mM dA, dG, dC and dUTP, 2 mM $MgCl_2$, 0.625 U of iTaq DNA polymerase, 250 nM of the forward and reverse primer, 5 μl of a 1:10 dilution of the cDNA, water was added to make 25 μl. The reactions were performed in the 96-well PCR plate covered with optical quality sealing tape. The reactions were performed for 40 cycles of 30 s at 95°C, 30 s at 58°C and 30 s at 72°C.

The end point used in the real-time PCR quantification, C_t, was defined as the PCR cycle number that crosses an arbitrarily placed signal threshold. Water was used as a negative control. The amount of leptin mRNA relative to β-actin was calculated as $2^{-\Delta C_t}$ where $C_t = C_{tleptinS} - C_{tActin}$. As relative expression of leptin gene in Ad-36 infected and uninfected controls was being studied and since the genes amplified at the same rate, no standard was used for absolute quantification.

Experiment 2. Leptin release by 3T3-L1 cells

(a) Leptin measurement: Differentiation of confluent 3T3-L1 preadipocytes (6 wells/group) was induced by MDI following their inoculation with Ad-36 or media (control) as described above. On day 5 post-inoculation, the supernatant media from all the wells was removed and diluted 1:2 with assay buffer provided with the kit (Titerzyme EIA mouse Leptin ELISA kit, Assay Designs Inc, Cat # 900-019). Leptin levels in the media were determined on day 5 by incubating the diluted media with mouse leptin antibody coated in 96-well plates for 1 h at 37°C. The wells were washed thoroughly with wash buffer and the leptin-primary antibody complex was incubated with a secondary antibody (rabbit IgG linked to horseradish peroxidase enzyme) for 30 min at 37°C. After thorough washing, all the wells were incubated with a substrate solution (tetramethyl benzidine) for the horseradish peroxidase and incubated for 30 min at room temperature in the dark. The Optical density was measured at 450 nm in a micro-plate reader. The standard curve was constructed using the standard leptin provided in the kit. Leptin release in the media was normalized to the lipid content of the well, which was determined as follows.

(b) Determination of cellular lipid content: Oil Red O, a lipid specific dye stains fat cells in proportion to the accumulated lipids and the assay is widely used to determine cellular lipid content.[25] After removing media for leptin determination as described above, the cells were fixed for 1 h with 10% formalin, then washed with water and stained for 2 h with Oil Red O, followed by exhaustive rinsing with water. After evaporating the excess water at 32°C the dye was extracted with isopropyl alcohol and its absorbance was determined at 510 nm, which was normalized to the amount of cellular DNA in the sample. The cellular DNA of each well was determined using the Wizard Genomic DNA Purification kit (Promega, Cat # A-1620).

Experiment 3: Effect of other human adenoviruses on differentiation and leptin secretion by 3T3-L1 cells

We determined if the effect of Ad-36 on differentiation and leptin secretion by 3T3-L1 cells was common to other subgroup D adenoviruses such as Ad-37 and Ad-9. Considering the fact that Ad-2, a subgroup C adenovirus does not induce preadipocyte differentiation[11] it was used as a negative control, whereas Ad-36 was used as a positive control. Confluent 3T3-l1 cells were inoculated with either media (control), Ad-36 (MOI 3.0), Ad-9 (MOI 1.0), Ad-37 (MOI 30) or Ad-2 (MOI 2.5) and differentiated as described in Experiment 1. Lipid accumulation in was determined by Oil Red O method and leptin in the media was measured by Titerzyme E1A mouse leptin ELISA kit (Assay Design, cat # 900-019) as described in Experiment 2. Lipid accumulation was normalized to the amount of DNA and leptin was normalized to the amount of lipid in each well.

Experiment 4 Leptin release from rat primary adipocytes

(a) Adipocyte isolation and culture. The animal protocols were approved by the Administrative Animal Care and Use Committee at University of California. Adipocytes were obtained from epididymal fat pads of male Sprague–Dawley rats (Harlan Sprague Dawley, Indianapolis, IN, USA) as previously described.[26] A brief description of the procedure is as follows.

Adipocyte isolation and culture Adipocytes were isolated from epididymal fat depots. Fat was minced with scissors in HEPES buffer (pH 7.4; containing 5 mM D-glucose, 2% BSA,

135 mM NaCl, 2.2 mM $CaCl_2 \cdot 2H_2O$, 1.25 mM $MgSO_4 \cdot 7H_2O$, 0.45 mM KH_2PO_4, 2.17 mM Na_2HPO_4 and 10 mM HEPES). Adipose tissue fragments were digested in the same buffer with type I collagenase (1.25 mg/ml per 0.5 g tissue) at 37°C with gentle shaking for 30 min. The resulting cell suspension was diluted in HEPES-phosphate buffer, and the isolated adipocytes were then separated from the undigested tissue by filtration through a 400-μm nylon mesh and washed three times. Isolated adipocytes were then re-suspended in DMEM supplemented with 1% FBS and incubated for 40 min at 37°C. The isolated adipocytes (150 μl of 2:1 ratio of packed cells to medium) were then plated on 500 μl of a collagen matrix (Vitrogen 100, Cohesion Technologies) in six-well culture plates. After 50 min of incubation at 37°C, 2 ml DMEM supplemented with 1% FBS was added to each well.

All six wells in one culture plate were seeded at equal density with the adipocytes isolated from one animal and inoculated with 4×10^5 PFU Ad-36, or media ($n = 7$ plates per group). After 24 h, the inoculation media was removed and replaced with 2 ml media containing no insulin or physiological concentration of insulin (0.48 or 1.6 nM). Within each plate, there were adipocytes treated without insulin, with insulin, with Ad-36, and with Ad-36 and insulin. The cells were maintained in an incubator at 37°C in 5% CO_2 for up to 96 h. Aliquots (300 μl) of the media were collected at 24, 48, 72, and 96 h and the sampled quantity was replaced with fresh medium. Glucose uptake, lactate production, and leptin secretion were determined.

(b) Assays

Leptin concentrations in the medium were determined with a sensitive and specific RIA for rat leptin (Linco Research, St Charles, MO, USA).[27] The antibody used in the assay does not cross-react with insulin. Glucose and lactate in the medium were measured with an YSI glucose analyzer (model 2300, Yellow Springs Instruments, Yellow Springs, OH, USA). Glucose uptake and lactate and leptin production were calculated as previously described.[28]

Experiment 5: Leptin, FAS and ACC-1 gene expression in rat visceral fat

(a) Experimental design. Male Wistar rats (5 weeks old) were obtained from Harlan (Indianapolis, IN, USA) and housed in micro isolator cages under bio-safety level two containment, with a separate air supply to each room. Protective clothing, gloves, shoes, hairnets and masks were used to enter the rooms and care was taken to prevent cross contamination. After 1 week of acclimatization to the new surroundings, rats were randomized in two weight-matched groups (control and infected) of 25 each and housed in separate rooms. Next the control and infected groups were i.p. injected with 200 μl of cell culture media or Ad-36 virus suspension ($\sim 10^8$ PFU), respectively. Access to food (chow diet) and water was provided *ad libitum* throughout the study and food consumption was recorded for individual cages. Rats were weighted weekly. After an overnight fast, animals were exsanguinated and killed 30 weeks post-inoculation, under Pentobarbital anesthesia. Epididymal-inguinal, retroperitoneal, and visceral fat depots were carefully separated, weighed and flash frozen in liquid Nitrogen. As we reported earlier,[29] infected rats had greater body weight and total fat compared to the control group (mean $\pm$ s.e.: 628.3 $\pm$ 13.0 g vs 587.5 $\pm$ 8.48 g; $P < 0.008$ and 52.3 $\pm$ 10.6 g vs 32.7 $\pm$ 3.5 g; $P < 0.00002$, respectively). Epididymal and inguinal fat pad, retroperitoneal fat pad and visceral fat pads were 60, 46 and 86% greater for the infected group compared with control.[29] Adipose tissue samples from these rats were used in the current experiment to determine the expressions of leptin, FAS and acetyl co-A carboxylase-1 (ACC1) in visceral adipose tissue of the two groups by quantitative real-time PCR assay (qRT-PCR). To minimize the confounding effect of different degree of adiposity on gene expression, three pairs of animals were selected from the two groups that matched closely in body weights and visceral fat weights. The percentiles for body weights for these animals were 30th, 50th and 75th from the control group, which were compared respectively, to the 20th, 30th or the 37th percentile from the infected group.

RNA extraction

RNA was extracted using the RNeasy Lipid Tissue Mini Kit, including treatment with DNase I to prevent genomic DNA contamination, strictly following the manufacturer's instructions (Cat # 74804, Qiagen, Valencia, CA, USA). Extracted RNA was stored at −80°C until required for cDNA synthesis.

Real-time PCR was performed as described for experiment 1. The sequence of the leptin primers was 5′-CACGA CACCCTTCACCACAAG-3′, forward primer and 5′-TTGAGG CAGAAGCTCCTCCA-3′, reverse primer.[30] FAS primers used were: forward: 5′-AGATGAAGGTGGTGGAGGTG-3′ and reverse 5′-TGCAGCTTGGTCTGAACATC-3′. ACC1 primers used were: forward 5′-ACCTTACTGCCATTCCATGC-3′and reverse: 5′-AAGCTTCCTTCGTGACCAGA-3′. Leptin primers used were: forward 5′-CACGACACCCTTCACCACAAG-3′ and Reverse 5′-TTGAGGCAGAAGCTCCTCCA-3′).

Statistical analysis

The effects of Ad-36 were determined by comparing the group means with those of the uninfected controls, using Student's *t*-test. Student's *t*-test with Simes correction was used for multiple comparisons. All values were expressed as means $\pm$ s.e.m. Values of $P < 0.05$ were considered significant.

Results

Experiment 1. Leptin gene expression

As expected, leptin mRNA was not detectable on 0, 1 and 2 days post-inoculation in either control or Ad-36 inoculated

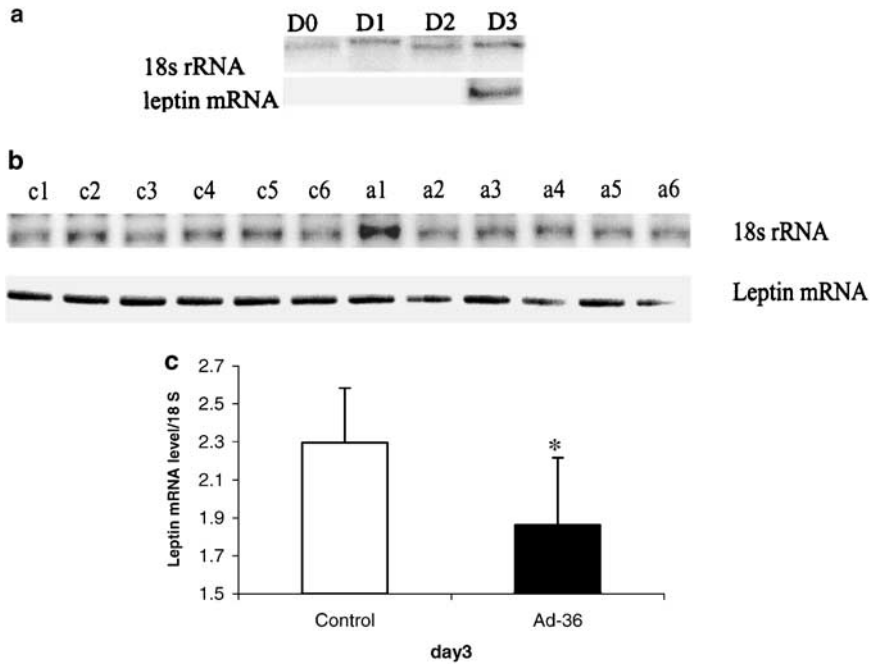

Figure 1 RNA was isolated on days 0, 1, 2 and 3 from uninfected control and Ad-36 infected 3T3-L1 cells. Leptin expression was determined using qualitative RT-PCR and results were normalized to 18 s rRNA levels. (**a**) Although 18 s rRNA was expressed on days 1, 2 and 3, leptin mRNA expression started on day 3. (**b**) 18 s rRNA and corresponding leptin expression on day 3. C1 to C6 and a1 to a6 are control and Ad-36 infected samples, respectively. (**c**) Comparison of leptin mRNA expression on day 3 for Ad-36 vs the uninfected control, quantitated by a Bio-Rad chemimager. *$P<0.05$ compared to the uninfected control group.

groups.[14] An expected rise in leptin mRNA expression was observed on day 3. Leptin mRNA expression on day 3 was 19% lower in the Ad-36 inoculated group ($P<0.05$) as determined by the qualitative RT-PCR method (Figure 1). Quantitative real-time RT-PCR analysis showed that Ad-36 suppressed leptin gene expression by approximately 58% on day 3 ($P<0.02$) and by 52% on day 5 ($P<0.04$) compared to the Control group (Figure 2).

Experiment 2. Leptin release by 3T3-L1 cells

As previously reported,[11] lipid accumulation normalized to the amount of DNA was significantly greater in the Ad-36 infected group (Figure 3). Leptin released in the media, unadjusted for lipid levels for the control and Ad-36 groups were 0.11 ± 0.02 vs 0.11 ± 0.01 absorbance at 450 nm, $P=0.7$, respectively. However, when normalized to the lipid content of the well, the leptin release was reduced to $51\pm8\%$ ($P<0.002$), in the Ad-36 group, compared to the control (Figure 3).

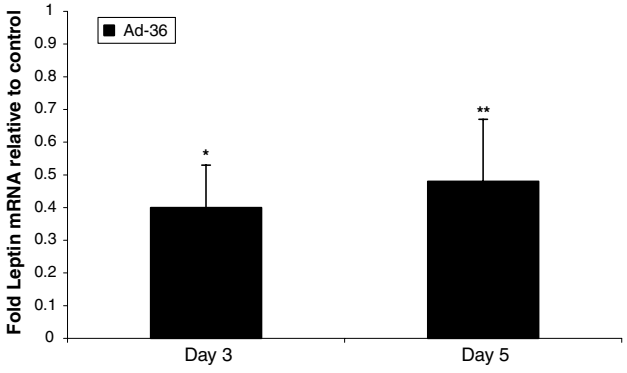

Figure 2 RNA was isolated on days 0, 2, and 3 from control and Ad-36 infected plates and tested for leptin expression using real-time RT-PCR and results were normalized to constitutive β-actin control. The percent leptin expression is shown relative to expression by uninfected control cells, which is considered 1. No leptin expression was detected on day 0 in control and infected samples. Ad-36 suppressed leptin gene expression by approximately 58% on day 3 (*$P<0.02$) and by 52% on day 5 (**$P<0.04$) compared to the Control group.

Experiment 3. Effect of other human adenoviruses on differentiation and leptin secretion of 3T3-L1 cells

Compared to the uninoculated control, subgroup D viruses Ad-36, Ad-37 and Ad-9 induced significantly greater lipid accumulation ($P<0.0001$; Figure 4a). When the leptin secretion was corrected for lipid content, Ad-36, Ad-37 and Ad-9 inoculated groups had lower leptin secretion ($P<0.002$; Figure 4b) on day 5 post inoculation. Ad-2, a Subgroup C virus did not share these effects (Figure 4).

Experiment 4. Leptin release by rat primary adipocytes

Leptin release by primary adipocytes from the epididymal fat pads of male Sprague-Dawley rats, inoculated with Ad-36, or control media, was not different in the absence of insulin, (Mean ± s.e.) 14.4 ± 5.0 vs 17.3 ± 6.4 ng, P = NS, (Figure 5). However, leptin release over 96 h was significantly lower in cells exposed to Ad-36 in presence of 0.48 nM insulin (21.5 ± 12.2 vs 34.1 ± 13.6 ng: $P<0.01$) or 1.6 nM insulin (19.3 ± 5.2 vs 32.6 ± 10.2 ng: $P<0.05$) (Figure 5). On the other hand, adipocytes inoculated with Ad-36 had greater glucose uptake in the absence of insulin (1233 ± 112 vs 636 ± 54 μg: $P<0.001$) and in the presence 0.48 nM insulin (1627 ± 82 vs 1389 ± 97 μg: $P<0.05$), but not in the presence of 1.6 nM insulin (2078 ± 55 vs 1931 ± 71 μg: P = NS) (Figures 6). Both lactate production (Figure 7) and the percentage of glucose metabolized to lactate were significantly higher in Ad-36 infected cells compared to uninfected cells (25.9 ± 3.2 vs 19.7 ± 3.0%, $P<0.001$; 25.5 ± 2.9 vs 17.2 ± 3.0%, $P<0.01$; 27.0 ± 3.9 vs 20.5 ± 2.8%, $P<0.05$ at 0, 0.48, 1.6 nM insulin concentrations, respectively).

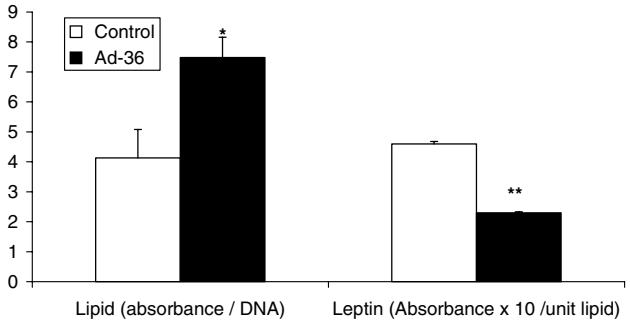

Figure 3 Uninfected control and Ad-36 infected 3T3-L1 cells were differentiated and on day 5 post-infection lipid accumulations in both groups were determined using the Oil Red O method. Leptin secreted in to the media was determined by ELISA and expressed as absorbance per unit lipid. Lipid accumulation normalized to the amount of DNA was significantly greater in the Ad-36 infected group (*$P<0.05$). Leptin release normalized to the lipid content of the well was reduced to 51 ± 8% (**$P<0.002$), in the Ad-36 group, compared to the control.

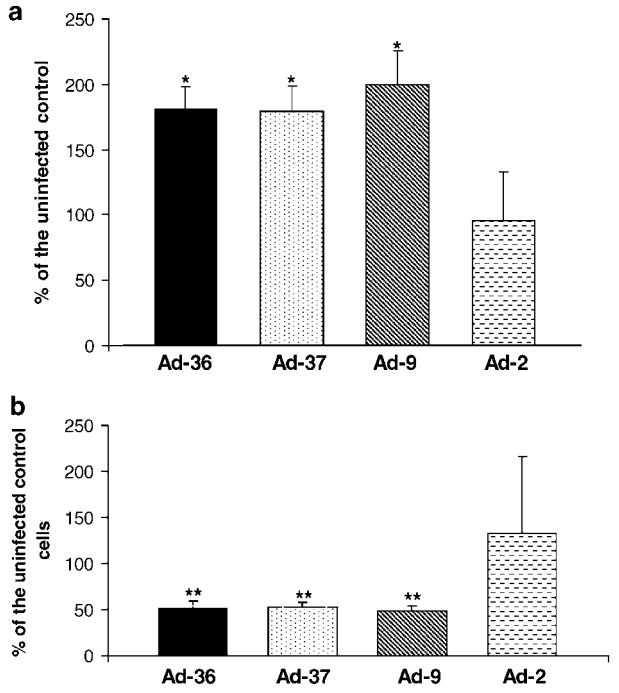

Figure 4 Lipid content and leptin secretion in 3T3-L1 cells infected with human adenoviruses. (**a**) 3T3-L1 cells were inoculated with media, Ad-36, Ad-37, Ad-9 or Ad-2, and differentiated with MDI. Lipid accumulation was determined 5 days post-inoculation using the Oil Red O method, normalized to the amount of cellular DNA in a sample and expressed as % of the uninfected control. *$P<0.0001$, compared to the uninfected control group. (**b**) 3T3-L1 cells were inoculated with media, Ad-37, Ad-37, Ad-9 or Ad-2, differentiated with MDI. Leptin secreted in to the media on day 5 was determined by ELISA and expressed as absorbance per unit lipid. **$P<0.002$, compared to the uninfected control group.

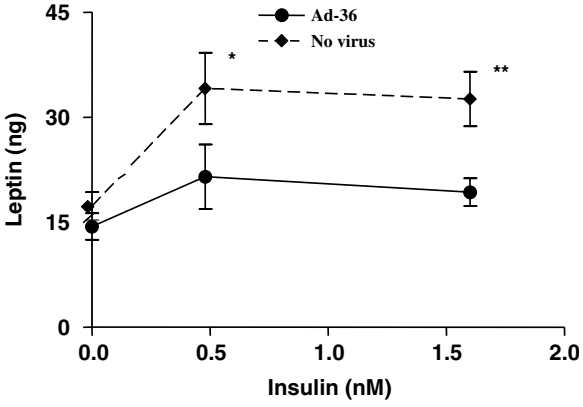

Figure 5 Leptin secretion over 96 h by cultured rat primary adipocytes pre-incubated for 24 h in the presence and absence of Ad-36. Data expressed as mean ± s.e.m. *$P<0.05$, **$P<0.01$ Ad-36 vs No virus, $n=7$.

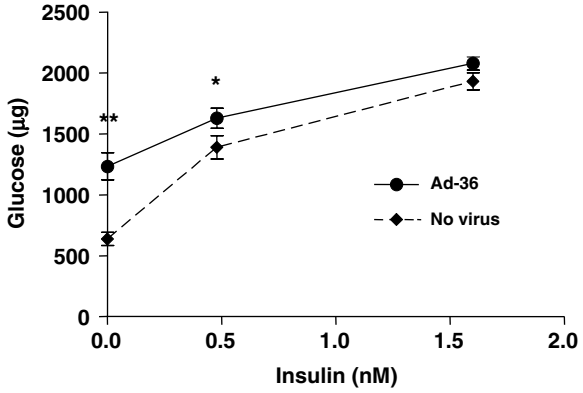

Figure 6 Glucose utilization over 96 h in cultured rat primary adipocytes pre-incubated for 24 h in the presence and absence of Ad-36. Data expressed as mean ± s.e.m. **$P<0.01$, ***$P<0.001$ Ad-36 vs No Adenovirus, $n=7$.

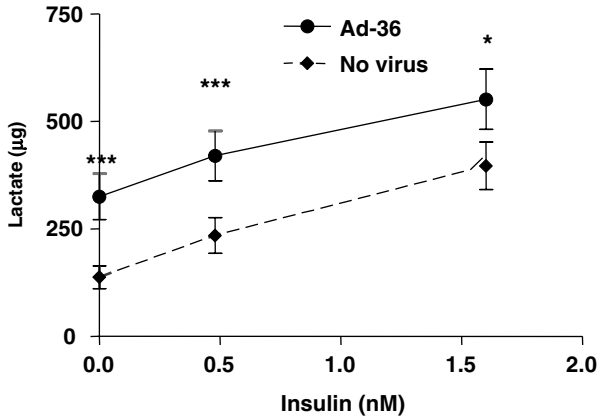

Figure 7 Lactate production over 96 h in cultured rat primary adipocytes pre-incubated for 24 h in the presence and absence of Ad-36. Data expressed as mean ± s.e.m. *$P<0.05$, ***$P<0.001$ Ad-36 vs No virus, $n=7$.

Table 1 Leptin, FAS and ACC-1 expression in rat visceral fat

	Pair 1		*Pair 2*		*Pair 3*	
	Control	*Infected*	*Control*	*Infected*	*Control*	*Infected*
Body weight (g)	567	569	613	612	591	598
Visceral fat	7.9	8.1	9.3	11.3	10.7	11.7
Leptin	1.0	0.2	1.0	0.6	1.0	0.5
FAS	1.0	1.23	1.0	6.34	1.0	2.19
ACC-1	1.0	1.58	1.0	4.0	1.0	21.11

One group of male Wistar rats were infected with Ad-36 (Infected group) and the other group was uninfected control. Animals were sacrificed 7 months later as reported earlier.[29] To minimize the confounding effect of different degree of adiposity on gene expressions, 3 pairs of animals were selected from the two groups that matched closely in body weights and visceral fat weights. Expressions of leptin, FAS and ACC-1 were determined by qRT-PCR, normalized to β-actin and expressed as a fold difference in the Infected animals compared to the Control. Expression in the Control group was considered to be 1. Body weight and visceral fat weights are at the time of killing. In rats from the infected group, leptin expression showed a significant decrease ($P=0.026$), where as the increase in ACC-1 and FAS did not reach statistical significance ($P>0.05$).

Experiment 5: Leptin, FAS and ACC1 expression in rat visceral fat

Leptin expression was 0.2, 0.6 and 0.5 fold lower in infected compared to the control rats that were matched for body weight and visceral fat weight, 30 weeks post-inoculation (Table 1). Interestingly, in the same pairs FAS and ACC1 expression was increased in the Ad-36 infected rats, indicating increased *de novo* lipogenesis despite similar body fat.

Discussion

Ad-36 is the first human adenovirus reported that causes obesity in experimentally infected animals and shows association with human obesity. It was first isolated from fecal sample of a diabetic girl suffering from enteritis.[31] In the United States, 30% of the obese but only 11% of the non-obese subjects showed antibodies to Ad-36. Although a human pathogen, Ad-36 is remarkably unselective about its choice of host. We have reported experimental infection of chickens, mice, rats and marmosets[6–8,29] and natural infection in rhesus monkeys[8] with Ad-36. We have reported transmission of Ad-36 infection by blood transfusion.[6] Not much more is known about the transmission routes of the virus. In experimentally infected animals, Ad-36 increases adiposity, without other overt signs and symptoms.

Mechanism of Ad-36 induced adiposity is unknown. Promotion of preadipocyte differentiation by Ad-36[11] may contribute to its adipogenic effect, a finding that prompted us to investigate consequential effects of the virus-induced differentiation on key metabolic and biochemical functions of adipocytes. This study focused on the effect of Ad-36 on *in vitro* expression and secretion of leptin, and the related modulations in glucose handling by the adipocytes.

Like primary adipocytes, leptin is also expressed in and released by 3T3-L1 cells (murine-embryo-derived cell line),[32] albeit in low quantities and the cell line has been used for studying the regulation of leptin expression and secretion.[33–35] Similar to the observations of others,[34] we also observed leptin expression 3 days after induction of differentiation of 3T3-L1 preadipocytes. Quantitative RT-PCR confirmed that leptin mRNA expression is lower in the virus-infected group. We hypothesized that the observed reduction in leptin mRNA would result in reduced leptin protein secretion into the media, which was determined in Experiment 2.

Rates of leptin secretion *in vitro* have been shown to strongly correlate with leptin mRNA levels ($r=0.89$, $P<0.001$).[36] Considering the continued relative suppression of leptin expression by Ad-36 on day 5 (Figure 2), and to allow its adequate production and release, leptin release by 3T3-L1 cells was determined on day 5 post-inoculation (Experiment 2). Leptin levels were within the detectable range of the standard curve.

Leptin released in the media was adjusted to the lipid content of the wells for the following reasons. It is well known that leptin levels strongly correlate with the amount of lipid[37,38] and its release is proportional to adipocyte volume.[36] Compared to the uninfected control, the virus increases differentiation of 3T3-L1 cells,[11] resulting in greater proportion of adipocytes and the lipid content. As adipocytes, but not preadipocytes secrete leptin, it would be misleading to compare leptin secretion of two groups containing unequal number or size of adipocytes. Therefore, as previously practiced by others,[39] leptin secretion was normalized to the amount of cellular lipid. Experiment 3 used rat primary adipocytes seeded at equal density. Therefore, there was no need to normalize leptin secretion to adipocytes in this experiment. Even without normalization for lipid content, leptin secretion in the virus inoculated group was lower in presence of insulin.

Consistent and complementary results were obtained in these independently conducted studies (Experiments 1 and 2), which collectively demonstrated that leptin expression and secretion were inhibited in cells infected with Ad-36. It is noteworthy that only (an unknown) fraction of the cells from Ad-36 group is likely to be infected. Apparently, the modulation induced by Ad-36 in infected cells is robust enough to produce significant reductions of leptin expression and secretion.

Although reduced leptin expression and secretion in presence of increased adiposity is an apparent paradox, our findings are very similar to those recently reported by Guo *et al*.,[40] who showed that genetic, diet-induced or age-related obesity is associated with decreased leptin gene expression per unit fat mass, compared to their respective lean counterparts. Lower plasma leptin concentrations per unit fat mass have been reported in humans as well.[41,42] Adenovirus-vector induced hyperleptinemia in rats depletes body fat.[19] Conversely, adipocyte-selective reduction of leptin receptors leads to adiposity in mice, despite a normal level of hypothalamic leptin receptors and normal food intake.[16] Therefore, we postulate that a relative hypoleptinemia may contribute to enhanced preadipocyte differentiation and lipid accumulation in Ad-36 infected rats.

Atkinson *et al*.[43] showed that Ad-37, but not Ad-2 increases adiposity in animals. Ad-36, Ad-37 and Ad-9 belong to subgroup D, whereas Ad-2 is a subgroup C human adenovirus. Experiment 3 determined if increased lipid accumulation and decreased leptin secretion is a subgroup D characteristic. All subgroup D adenoviruses tested increased lipid accumulation in 3T3-L1 cells, but reduced leptin secretion. It appears that viruses that increase adiposity in animal models (Ad-36 and Ad-37) modulate lipid accumulation and leptin secretion by adipocytes, which may be contributing to their adipogenic effect observed *in vivo*. Whereas Ad-2, a non-adipogenic virus, does not share this property. These suggested that the adipogenic potential of Ad-36, Ad-37 and Ad-9 could be a subgroup characteristic. Also, the results demonstrated that adipogenic and hypoleptinemic properties are not common to all human adenoviruses.

Considering the close relationship of leptin with glucose uptake and metabolism,[26,28] Experiment 4, investigated the effect of Ad-36 on glucose uptake. Adipocytes, instead of preadipocytes were used to obtain a sizable effect on glucose uptake. Ad-36 affected adipocyte glucose metabolism; increased glucose uptake, lactate production and the percent of glucose metabolized to lactate. Decrease in leptin production was not due to shutting down of cellular metabolic activity due to viral infection. In fact, increased cellular metabolic activity was evidenced from increased glucose uptake and the lactate levels. Moreover, the effect was not due to desensitization of cells to insulin during the incubation period. In a dose–response comparison using physiologically relevant concentrations of insulin used in this study, the rates of glucose uptake and leptin secretion increase more steeply during the final 48 h of culture compared to the first 48 h (data not presented). The same was true for glucose uptake and leptin secretion from the adipocytes cultured with and without Ad-36 in presence of 1.6 nM insulin (data not presented).

Effects of insulin to stimulate leptin gene transcription, mRNA expression, and secretion are mediated by the increases of adipocyte glucose utilization,[26,28] and more specifically to its effect to shift the metabolism of glucose away from anaerobic metabolism to lactate.[44] Thus, certain agents that increase the uptake of glucose, can concurrently induce an inhibition of leptin secretion from cultured primary adipocytes, if the compound also increases the proportion of glucose anaerobically metabolized to lactate.[44] Therefore, it is possible that inhibition of insulin-mediated leptin secretion induced by Ad-36 was a result of its effect to increase anaerobic glucose metabolism. However, there was no statistical relationship between the decrease in leptin secretion and increase in lactate production or percentage of glucose metabolized to lactate induced by Ad-36 within each dose of insulin or across all doses ($r = 0.02$–0.20, $P = 0.4$–0.9). Thus, these data suggest that the inhibition of leptin production induced by Ad-36 is independent of its effects on adipocyte metabolism. It is possible that Ad-36 infection induces a direct inhibition of leptin gene transcription.

The mechanism of modulation of leptin and glucose metabolism by Ad-36 unclear. This study was not designed to determine whether reduced leptin production has a causal role in promoting *in vitro* adipogenesis. It is conceivable that a relative decrease in leptin in the Ad-36 infected cells leads to attenuation of the usual lipolytic effects of leptin, resulting in increased preadipocyte differentiation and lipid accumulation. Furthermore, it is possible that increased glucose uptake by Ad-36 infected adipocytes could contribute to greater lipid accumulation if, it also increases *de novo* lipogenesis. Attenuation of leptin production in Ad-36 infected animals may also lead to increased adiposity through increased food intake, decreased energy expenditure or both.[12,45] Effect of CDV (Canine Distemper virus), an adipogenic virus, on leptin network has been previously reported by Bernard *et al*.[46] Expression of the long leptin receptor isoform, was downregulated in the hypothalami of obese mice,[46] which is postulated to contribute to CDV-induced adiposity. Effect, if any, of Ad-36 on hypothalamic leptin receptors is unknown. Unlike Ad-36, CDV primarily affects the CNS. Previous experiments did not show any overt hypothalamic lesions in Ad-36-infected animals (Dhurandhar *et al*., unpublished observations). Therefore, this study focused on peripheral effects of Ad-36.

Experiment 5 was conducted to obtain preliminary evidence for the effects of Ad-36 infection on leptin and adipose glucose metabolism ex *vivo*. Earlier, we reported increased adiposity in male Wistar rats infected with Ad-36.[29] We used adipose tissue from this rat study for Experiment 5 to determine the effect of Ad-36 on expression of leptin and genes of *de novo* lipogenesis. As the amount of

circulating leptin is proportional to body fat, ideally such a comparison should be made between Ad-36 infected and uninfected animals of similar body fat content. Therefore, we compared three pairs from the two groups, matched as closely as possible for adiposity and body weights. Reductions in adipose tissue leptin expression and enhanced expression of genes of *de novo* lipogenic pathway observed in this *ex vivo* experiment (Experiment 5) are in congruence with the *in vitro* data. It is possible that Ad-36 promotes increased glucose uptake in adipocytes, which is converted to lipids via the upregulated *de novo* lipogenic pathway. Vector-induced hyperleptinemia reduces ACC-1 and FAS mRNA.[47] Therefore, the upregulation of ACC-1 and FAS in presence of relative hypoleptinemia may be expected.

It should be pointed out that circulating leptin levels of rats described in Experiment 5[29] were significantly greater for the Ad-36-infected group. Reduction in adipose tissue leptin expression but greater circulating leptin seems paradoxical. However, it should be noted that the Ad-36-infected rats had significantly greater amount of fat ([29] 52 ± 11 vs 33 ± 6 g, $P < 0.00001$). It is likely that the net effect of greater adiposity is increased plasma leptin levels compared to the lean group, despite reduced leptin expression per unit adipose tissue. In future, it would be important to compare circulating leptin levels of Ad-36-infected animals with those with similar degree of adiposity.

Circulating leptin levels have not been examined in humans who are sero-positive for Ad-36. Subjects with relative hypoleptinemia have lower-serum cholesterol compared to relatively hyperleptinemic subjects with similar BMI.[48] Similarly, it is noteworthy that relative hypocholesterolemia is caused by Ad-36 in animal models[6–8] and it is associated with the presence of Ad-36 antibodies in humans.[10] It is unknown whether subjects with relative hypoleptenemia and hypocholesterolemia[48] have a higher prevalence of sero-passivity for Ad-36. In such studies, it would be important to compare the leptin levels in Ad-36 sero-positive and control subjects with comparable adiposity and patterns of body fat distribution.

In conclusion, these *in vitro* experiments demonstrate that Ad-36 downregulates leptin expression and secretion in 3T3-L1 cells, as well as leptin secretion from primary adipocytes. Furthermore, the data suggest that Ad-36 increases *de novo* lipogenesis in adipocytes by Ad-36. Future studies should investigate whether an inhibition of leptin production contributes to enhanced adipogenesis or positive energy balance and therefore to obesity in Ad-36 exposed animals and humans.

Acknowledgements

This work was supported in part by funds from the William Hardy Endowment for Obesity Research, and NIH Grant 1R01 DK066164-01 to NVD.

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