Adenovirus infection results in alterations of insulin signaling and glucose homeostasis

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Jiang S, Gavrikova T, Pereboev A, Messina JL. Adenovirus infection results in alterations of insulin signaling and glucose homeostasis. Am J Physiol Endocrinol Metab 298: E1295–E1304, 2010. — Recombinant adenovirus (Ad) vectors can initiate an inflammatory response, limiting its use in gene therapy and basic research. Despite increased efforts to better understand Ad infection, little is known about how it affects cellular metabolic responses. In the current studies, we explored the effects of Ad vectors on insulin signaling molecules and glucose homeostasis. Nonreplicative Ad vectors were injected into rats through the tail vein, and at 4–13 days postinjection insulin signaling and glucose tolerance were examined. Ad vector infection significantly reduced total levels of the insulin receptor (IR), and insulin receptor substrates 1 and 2 (IRS-1, IRS-2) in the liver of rats, resulting in decreased insulin-induced tyrosine phosphorylation of IR, IRS-1, and IRS-2, and decreased interaction of IRS-1 and IRS-2 with phosphoinositide 3-kinase (PI3K). In addition, Ad infection resulted in impaired systemic glucose homeostasis, which recovered by 13 days, after the protein levels of IR, IRS-1, and IRS-2 had started to normalize. Expression of a TNF inhibitor or Kupffer cell depletion attenuated the Ad vector-induced decreases of insulin signaling molecules, indicating a potential role of Kupffer cell activation in this process. These studies provide evidence that systemic administration of Ad vectors can impair insulin signaling in liver, resulting in altered systemic glucose metabolism. Thus, effects of Ad vector infection on insulin action and glucose metabolism need to be considered when Ad vectors are used in research or gene therapy and may be more broadly applicable to other viral agents.

liver; insulin receptor; insulin receptor substrates; tumor necrosis factor-α; Kupffer cells

RECOMBINANT ADENOVIRUS (AD) VECTORS are widely used as gene delivery vehicles in human gene therapy clinical trials and basic research experiments. Although the Ad vectors are highly efficient as a gene transfer tool, the use of these vectors is not without problems. For human gene therapy, the acute host immune response to Ad vectors can cause injury of infected tissues (29) and can result in death (24). For basic research experiments, cellular changes induced by the Ad vector itself may confound the roles of certain transgenes and complicate data interpretation (26). It is therefore important to understand all aspects of Ad vector infection and its related toxicity (18, 26, 29, 33, 34). Inflammation induced by viral infection, such as by hepatitis C and hepatitis B viruses, has been linked with the development of insulin resistance and type 2 diabetes (6, 12, 14, 35). However, it is not known whether Ad vector infection can affect insulin signaling and glucose metabolism.

First-generation E1/E3-deleted Ad vectors were derived from wild-type Adenoviridae, and are replication incompetent. Despite the modifications, E1/E3-deleted Ad vectors still express low levels of the remaining viral genes (41). The Ad vector gene products and the Ad particles or capsid are known to cause immune responses (26, 39). Host responses induced by Ad vectors involve activation of inflammatory pathways and increased production of various cytokines, including TNFα (28, 29, 48). The majority of the Ad vectors are rapidly sequestered in the liver upon systemic administration (34). The liver’s resident macrophages, Kupffer cells, are activated and release cytokines and chemokines within the tissue to eliminate infected cells and play a role in the early liver immune response after intravenous injection of Ad vectors (17, 33). This is supported by the evidence that removal of Kupffer cells prior to Ad vector injection reduces release of TNFα and decreases liver injury (17, 45). Many inflammatory factors, including TNFα, can inhibit the activity of the insulin receptor (IR) and promote the ubiquitination and subsequent degradation of insulin receptor substrate proteins [IRSs (15, 32)]. Based on the role of inflammation in insulin resistance, our hypothesis is that systemic administration of Ad vectors leads to impaired insulin signaling due to activation of Kupffer cells and TNFα production.

Insulin signaling is initiated by the binding of insulin to its receptor on the surface of target cells, which results in auto-phosphorylation and activation of the IR (37). The activated IR then recruits and phosphorylates IRSs at multiple tyrosine residues. Tyrosine-phosphorylated IRS proteins act as docking sites for phosphoinositide 3 kinase (PI3K), which becomes activated and then activates downstream target proteins (37). In liver, IRS-1 and IRS-2 have complementary roles in insulin signaling (38), and IRS-2 is essential for PI3K activity and Akt activation by insulin in liver (38, 40). Decreased levels or inactivity of the IR or IRS proteins can decrease insulin signal transduction and lead to insulin resistance (37, 38).

In the present study, we found that systemic Ad vector administration decreased insulin-induced tyrosine phosphorylation of IR, IRS-1, and IRS-2 in the liver of rats, which was due to decreased total amounts of IR, IRS-1, and IRS-2 proteins. Systemic glucose homeostasis was also impaired by Ad vector infection as a result of a defect in hepatic insulin signaling. Expression of a TNFα inhibitor or Kupffer cell depletion prior to Ad vector injection attenuated Ad vector-induced defects in insulin signaling molecules, indicating a potential role of Kupffer cell activation-mediated local inflammation in these processes.
RESEARCH DESIGN AND METHODS

Ad vectors. Ad serotype 5 vectors, including Ad5-LacZ [encoding β-galactosidase (β-Gal)] and Ad5-TNFαi (encoding soluble TNF receptor-IgG heavy -chain chimERIC protein), were kindly provided by Dr. Jay Kolls (13). For the Ad5-LacZ, the LacZ cDNA fragment was cloned into the pACCMV plasmid [contains 0–1.3 map units (mu) of the Ad5 genome, the CMV promoter, and 9.0–17.0 mu of the Ad5 genome]. The pACCMV plasmid was cotransfected with the Ad backbone plasmid pJM17 (carries the entire Ad5 genome with a deletion in E3 and an insertion of prokaryotic DNA fragment in E1) into 293 cells to generate recombinant Ad5-LacZ. A second β-Gal-expressing vector (Ad5-nt-LacZ) was purchased from the University of Iowa Vector Core. The Ad5-nt-LacZ was constructed with the RAPAd.1 system (1), where the LacZ cDNA fragment was cloned into the pacAd5CMV-K-NpA plasmid (contains 0–1 mu of the Ad5 genome, the CMV promoter, and 9.2–16.1 mu of the Ad5 genome). The pacAd5CMV-K-NpA plasmid was cotransfected with the plasmid pacAd5(9.2–100)sub360 (devoid of the left-hand ITR, the packaging signal, and E1 sequences, but containing 9.2–100 mu of the Ad5 genome) into 293 cells to generate Ad5-nt-LacZ. The Ad vectors were amplified in 293 cells and purified twice by cesium chloride gradient ultracentrifugation followed by dialysis. The biological titer [plaque forming units (pfu)/ml] was determined by the TCID50 (tissue culture infectious dose 50) method (AdenoVactor Vector System) based on the development of a cytopathic effect in 293 cells. The viral particle titration was based on evaluation of particles in solution correlating to DNA content. For all virus preparations, the ratio of particles to infectious units was 10–20. The replication-competent Ad contamination was not detected in purified virus stocks as screened by PCR with the primers flanking the E1 region. For the UV-inactivated virus group, equal amounts of Ad5-LacZ (1010 pfu) were exposed overnight to UV light (254 nm).

Ad vector infection. All experimental procedures were carried out in accordance with the Guidelines of the Care and Use of Laboratory Animals by the National Institutes of Health, and the experimental protocols were approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham. Male Sprague-Dawley rats (200–250 g, Harlan) were housed in the animal facilities for at least 1 wk before experiments. The purified Ad vectors were injected into rats through the tail vein in 500 μl of PBS. At different time points (4–13 days) after injection, tissues were harvested or glucose tolerance tests were performed. For insulin signaling studies, the rats were fasted for 18–20 h before tissue harvesting, and insulin (5 U) or saline was injected through the portal vein. Tissues were harvested at 1 min after insulin or saline injection and quickly frozen in liquid nitrogen.

Surgical trauma procedures. The surgical trauma procedures were performed as described previously (16, 21, 22, 46). Rats were fasted for 18–20 h and then anesthetized by inhalation of 1.5% isoflurane, and a 5-cm midline laparotomy was performed. At 90 min (T90′) or 210 min (T210′) after the trauma procedure, insulin (5 U) or saline was injected through the portal vein, and tissues were harvested.

Western blotting. Liver, skeletal muscle, and fat tissues were homogenized in lysis buffer as described previously (16, 21, 22, 46). Protein concentrations were measured by BCA assay (Pierce, Rockford, IL). The lysates (30 μg/lane) were subjected to SDS-PAGE and transferred to nitrocellulose membranes. The membranes were incubated with anti-PY972-IR, anti-PY612-IRS-1 (Invitrogen, Carlsbad, CA), anti-total-IR, anti-total-IRS-1 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-total-ERK, anti-pan-actin, anti-epidermal growth factor receptor (EGFR), and anti-total-IRS-2 (Cell Signaling, Danvers, MA) antibodies, followed by horseradish peroxidase (HRP)-conjugated secondary antibody and detected by enhanced chemiluminescence (Pierce, Rockford, IL). The bands were analyzed by imaging densitometry.

Immunoprecipitation. Liver extract (1 mg) was incubated with antibody specific for IRS-1 or IRS-2 (Santa Cruz Biotechnology) overnight at 4°C with gentle agitation. Protein A beads were then added and incubated for 4 h at 4°C. Immunoprecipitated proteins were subjected to SDS-PAGE and transferred to nitrocellulose membranes. The membranes were incubated with anti-PI3K p85, anti-PUPI (Upstate Biotechnology, Lake Placid, NY), and anti-total-IRS-1 and -IRS-2 antibodies.

β-Gal staining. Liver sections were stained using a β-Gal staining kit (Mirus Bio) according to the manufacturer’s instructions. Sections of frozen liver (10 μm) were fixed in glutaraldehyde (2%, 5 min), and incubated overnight with X-Gal reagent at 37°C and then counterstained briefly with nuclear fast red. Images were obtained using a Leica microscope (Leica Microsystems).

Glucose tolerance tests. Glucose tolerance tests were performed after an overnight fast. Rats were injected intraperitoneally with 0.6 g/kg d-glucose (25%), and blood glucose was measured at 15-min intervals for up to 120 min with a glucose meter (TheraSense) by sampling of blood from a tail vein.

Gadolinium chloride treatment. Rats were treated with GdCl3 to deplete Kupffer cells as described previously (17). GdCl3 (Sigma-Aldrich) was dissolved in saline, and was injected (10 mg/kg body wt) via the tail vein at 30 min and 6 h prior to Ad administration. GdCl3 (10 mg/kg) was given again at 3 days after Ad vector injection, and the livers were harvested 6 days after Ad vector administration.

Immunohistochemistry. The liver tissues were rinsed in PBS and embedded in HistoPrep Frozen Tissue Embedding Media (Fisher Scientific) and snap-frozen in liquid nitrogen. Frozen livers were cryosectioned (10 μm) and subjected to immunohistochemical staining with anti-CD68 antibody (AbD Serotec). The liver sections were fixed in 70% ethanol overnight and blocked with 0.1% sodium azide and 0.5% H2O2 in PBS for 15 min. Overnight incubation with the primary antibody (Anti-CD68, 1:200 in 1% BSA) was followed by HRP-conjugated secondary antibody and DAB substrate. The sections were counterstained briefly with hematoxylin, and images were obtained using a Leica microscope (Leica Microsystems).

Statistical analysis. Data are presented as means ± SE. The statistical analysis was performed using GraphPad InStat software (San Diego, CA). Statistical differences between two groups were analyzed by Student’s t-test. For comparison between multiple groups, ANOVA was performed followed by Student-Newman-Keuls posttest. P < 0.05 was considered statistically significant.

RESULTS

Effects of Ad infection on insulin signaling. Previous work from our laboratory focused on the development of acute hepatic insulin resistance following surgical trauma and hemorrhage (16, 21, 22, 46). We wished to study the mechanisms underlying this acute onset of insulin resistance after injury using Ad as a gene transfer tool to express proteins in vivo. Our initial studies included injection of a replication-deficient Ad vector, encoding β-Gal (Ad5-LacZ, 1010 pfu) into rat via the tail vein. At 6 days after Ad5-LacZ injection, surgical trauma for 90 min (T90′) was performed, a normal part of our surgical procedure. Compared with the uninfected group, Ad5-LacZ injection significantly decreased insulin-induced phosphorylation of IR and IRS-1 (Fig. 1A). We then measured the total amounts of IR and IRS-1 in the liver of rats, and they were reduced as a result of Ad vector administration (Fig. 1B). These observations were further confirmed by our second group of experiments using new preparations of the same vector and examined in rats 210 min after trauma (Fig. 1C). An increase in the level of total ERK protein was also observed following Ad vector infection (Fig. 1C). Since our previous studies had
demonstrated that trauma alone had no effect on the total amount of IR and IRS-1 (21), the observed decreases in insulin signaling were likely due to infection with the Ad vector itself.

**Ad infection reduced total amounts of hepatic IR, IRS-1, and IRS-2.** To confirm that the observed decreases of insulin signaling molecules were induced by the Ad vector instead of the combination of Ad and surgical trauma, rats were injected with Ad5-LacZ, and tissues were harvested without the surgical trauma procedure being performed, and similar data were obtained. Infection with Ad vector decreased insulin-induced phosphorylation of IR and IRS-1, as well as the total levels of IR and IRS-1 (Fig. 2A). The level of EGFR was not affected by Ad vector infection (Fig. 2A) and indicated that all lanes were loaded evenly. In liver, IRS-2 is also an important insulin receptor substrate for insulin action. Similar to IRS-1, Ad vector infection decreased insulin-induced IRS-2 tyrosine phosphorylation in liver as well as the total amount of IRS-2 (Fig. 2B). Comparing the ratio of phosphorylated IR, IRS-1, or IRS-2 to the total amount of IR, IRS-1, or IRS-2, respectively, there was no significant difference between the Ad vector-infected and the uninfected rats (Fig. 2C). This suggests that the decreases in insulin-induced phosphorylation of IR, IRS-1, and IRS-2 in liver by Ad vector infection were entirely due to reductions in total amounts of IR, IRS-1, and IRS-2, respectively.

To determine how Ad vector affects insulin signaling downstream of IRS proteins, insulin-induced IRS-1/PI3K and IRS-2/PI3K interactions were examined by immunoprecipitation. Ad vector infection significantly decreased insulin-induced IRS-1/PI3K interaction in liver (Fig. 2D). Similarly, insulin-induced IRS-2/PI3K interaction was also suppressed following Ad vector infection (Fig. 2D). The total amount of PI3K p85 was not decreased following Ad vector injection compared with uninfected rats (Fig. 2D), suggesting that the decreased binding of PI3K was due to the decreased levels of IRS-1 and IRS-2.

**Time course of Ad vector-induced effects on protein levels of hepatic IR, IRS-1, and IRS-2.** To ensure that the Ad vector effects on insulin signaling were not due to one specific Ad vector, a second E1/E3 deleted Ad vector, Ad5-nt-LacZ, was used. Three different doses of Ad5-nt-LacZ (2 × 10¹⁰, 1 × 10¹⁰, and 5 × 10⁹ pfu per animal) were injected, and tissues

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**Fig. 1.** Adenovirus (Ad) vector infection decreased protein levels of insulin receptor (IR) and IRS-1 and their phosphorylation in liver of rats after surgical trauma. Rats were injected with Ad5-LacZ (1 × 10¹⁰ pfu) via tail vein; the uninfected group was without Ad vector injection. At 6 days postinjection, trauma for 90 min (A and B; n = 4 rats) or 210 min (C; n = 6 rats) was performed, insulin (+ins) or saline (−ins) was injected via portal vein, and liver tissue was harvested and subjected to Western analysis with anti-PY972-IR-, total-IR-, PY612-IRS-1-, total-IRS-1-, pan-actin-, and total-ERK-specific antibodies. Bands were scanned and quantified using ZeroD Scan. Bar graphs of PY972-IR (n = 3 rats each group), PY-612-IRS-1 (n = 3 rats each group), total-IR (n = 4 rats each group), and total-IRS-1 (n = 4 rats each group) are presented as means ± SE. T90’, trauma for 90 min; T210’, trauma for 210 min.
Fig. 2. Hepatic IR, IRS-1, IRS-2 and their phosphorylation, and insulin-induced interaction of IRS-1 and IRS-2 with phosphoinositide 3-kinase (PI3K) were reduced by Ad vector infection independently of surgical trauma. Rats were injected with Ad5-LacZ (1 × 10^10 pfu) via tail vein. A: at 6 days postinjection, liver tissue was harvested and subjected to Western blot (WB) analysis with PY972-IR, total-IR, PY612-IRS-1, total-IRS-1, and epidermal growth factor receptor (EGFR) antibodies. B: liver tissues were immunoprecipitated (IP) with total-IRS-2-specific antibody. Precipitates were subjected to WB with anti-tyrosine-specific antibody and total-IRS-2 antibody. Bands were scanned and quantified with ZeroD Scan. Bar graphs of PY972-IR (n = 3 rats each group), PY-612-IRS-1 (n = 3 rats each group), PY-IRS-2 (n = 3 rats each group), total-IR (n = 4 rats each group), total-IRS-1 (n = 4 rats each group), and total-IRS-2 (n = 4 rats each group) are presented as means ± SE. C: bar graphs of the ratio of phosphorylated to total levels of IR, IRS-1, and IRS-2. P ≥ 0.05 is considered not significantly different (NS). D: liver lysates were immunoprecipitated with total-IRS-1- or total-IRS-2-specific antibodies. Precipitates were subjected to SDS-PAGE gel and probed with anti-PI3K p85, total-IRS-1, and total-IRS-2 antibodies. Bands of PI3K p85 were scanned and quantified with ZeroD Scan. IB, immunoblot. Bar graphs of PI3K-IRS-1 (n = 3 rats/group) and PI3K-IRS-2 (n = 3 rats/group) are presented as means ± SE.
were harvested 4, 6, and 8 days postinjection. For the $1 \times 10^{10}$ pfu dose, two longer time points, 10 and 13 days, were also investigated. Injection of Ad5-nt-LacZ ($1 \times 10^{10}$ pfu) resulted in expression of β-Gal in most liver cells at 4 days, with expression decreasing with time (Fig. 3A). For all doses of Ad5-nt-LacZ, decreased IR, IRS-1, and IRS-2 were detected by 4 days postinjection (Fig. 3B). Thus, infection of rats with Ad vectors resulted in decreased hepatic IR, IRS-1, and IRS-2 levels. Despite the difference in doses of Ad vector and variability from rat to rat, the timing of the effects of Ad vector on IR and IRS-2 were quite similar, which started to decrease by 4–6 days, with at least partial recovery by 8 days. The decrease of IRS-1 protein was more severe and occurred earlier than IR and IRS-2, and recovery did not start until later.

Since IRS-1 serine phosphorylation is known to be a negative regulator of insulin signaling and can also lead to IRS-1 degradation (4, 10, 43, 47), phosphorylation of IRS-1 at Ser307 in liver after Ad vector infection was examined. An increase in IRS-1 serine phosphorylation was measurable at 4 days postinjection of Ad5-nt-LacZ with a dose of $2 \times 10^{10}$ pfu, but not at later time points (Fig. 3C), when the levels of IRS-1 were so low that they were sometimes difficult to measure without greatly overexposing the Western blot. This increase in IRS-1 serine phosphorylation after Ad vector infection could be involved in the decrease of total IRS-1 protein level.

No effects of Ad infection on skeletal muscle and adipose tissue. The effects of Ad vector on insulin signaling in two other insulin target tissues, skeletal muscle and adipose tissue, were examined. In contrast to what was found in liver, Ad vector infection did not alter the levels of total IR and IRS-1 and their insulin-induced tyrosine phosphorylation levels in triceps (Fig. 4A) and epididymal adipose tissue (Fig. 4B). Also, the amount of total-ERK in skeletal muscle and adipose tissue was not altered following Ad vector infection (Fig. 4, A and B). This indicates that Ad vector infection affects insulin signaling in liver but not skeletal muscle or adipose tissue, which is consistent with liver tropism after systemic administration of Ad vector.

Expression of TNF inhibitor attenuated effects of Ad vector injection. Previous studies indicate that systemic Ad vector administration can increase serum or hepatic TNFα levels more than 10-fold, with two peaks occurring at 3–5 h and 2–5 days, after which TNFα level returns to normal (17, 19). TNFα can promote the degradation of IRS proteins (15, 32). To investigate the role of TNFα in Ad vector infection-induced decreases in insulin signaling molecules, an Ad vector encoding a TNF inhibitor was injected into rats at the same dose as Ad5-LacZ ($1 \times 10^{10}$ pfu). Six days postinjection, expression of bioactive TNFα inhibitor in the blood of rats after Ad5-TNFi administration was verified using an in vitro TNFα toxicity bioassay (data not shown). Consistent with previous data (Figs. 1 and 2), Ad5-LacZ injection decreased the total amounts of hepatic IR and IRS-1 (Fig. 5). The decrease in IR by Ad5-LacZ was completely prevented by inhibition of TNFα toxicity as a result of TNF inhibitor expression (Fig. 5, A and B). For IRS-1, Ad5-TNFi administration resulted in decreased IRS-1 level compared with the uninfected group but was significantly higher than in the Ad5-LacZ group (Fig. 5, A and C), suggesting a partial reversal of the IRS-1 decrease by inhibition of...
TNFα. Thus, TNFα may play a role in Ad vector-induced defects in insulin signaling in liver.

It is known that the first-phase production of TNFα following Ad infection is a result of the innate immune response triggered by the viral particles, independent of viral gene transcription, as evidenced by increased TNFα after UV-inactivated Ad administration (19, 29). Thus, the effects of UV-inactivated Ad on insulin signaling molecules were examined. The protein levels of IR, IRS-1, and IRS-2 were slightly reduced by UV-inactivated Ad5-LacZ but much less than when active Ad vectors were used (Fig. 6A). This suggests that the early innate immune response triggered by viral particles, as well as the later response to viral gene expression and infectivity, both contribute to decreases in hepatic insulin signaling molecules.

Depletion of Kupffer cells by GdCl3 attenuated effects of Ad vector infection. Resident hepatic macrophages, Kupffer cells, are a significant source of TNFα following Ad vector administration and may play a role in Ad vector-induced inflammation in liver (17, 28, 33). To determine the role of Kupffer cells in Ad vector infection-induced defects in hepatic insulin signaling, rats were treated with GdCl3 to deplete Kupffer cells prior to Ad vector administration. To prevent the repopulation of Kupffer cells, rats were treated with GdCl3 again 3 days after Ad vector injection, and tissues were harvested at 6 days. Immunohistological staining with antibody specific for CD68, a Kupffer cell marker, confirmed that GdCl3 treatment largely decreased the number of Kupffer cells in liver (Fig. 6B). Depletion of Kupffer cells partially inhibited the Ad vector infection-induced decrease in the protein levels of total-IR, IRS-1, and ERK.

Fig. 4. Levels of total-IR and IRS-1 and their phosphorylation in skeletal muscle and adipose tissue were not affected by Ad vector. Rats were injected with Ad5-LacZ (1 × 1010 pfu) via tail vein. At 6 days postinjection, skeletal muscle (A) and adipose tissue (B) were harvested and subjected to Western analysis with PY972-IR, total-IR, PY612-IRS-1, total-IRS-1, and total-ERK antibodies. Bands were scanned and quantified using ZeroD Scan. Bar graphs of PY972-IR and PY-612-IRS-1 in triceps (A) and adipose tissue (B) are presented as means ± SE (n = 3 rats for all groups). P < 0.05 is considered NS.

Fig. 5. Levels of IR and IRS-1 in liver after Ad5-LacZ and Ad5-TNFi infection. In addition to uninfected and Ad5-LacZ-infected groups, described in figure legends 1 and 2, another group of rats (n = 14) was injected with an equal amount (1010 pfu) of Ad5-TNFi. At 6 days postinjection, tissues were harvested with surgical trauma procedure (4 rats, T90’, 6 rats, T210’, and 4 rats without surgical trauma). Protein levels of total hepatic IR, IRS-1, and ERK in all groups were examined by Western analysis (A). Bar graphs of total-IR (B) and total-IRS-1 (C) in liver are presented as means ± SE (n = 14 rats in each group, all including 4 rats with T90’, 6 rats with T210’, and 4 rats without surgical trauma). P < 0.05 is considered NS. White gap (A) indicates grouping of lanes from different parts of the same gel.
total-IRS-1, and total-IRS-2 (Fig. 6C). Treatment with GdCl$_3$ alone had no effect on the level of insulin signaling molecules (unpublished data), whereas the current data suggest that activation of Kupffer cells may be involved in the impairment of insulin signaling following Ad vector infection.

Ad infection resulted in decreased glucose tolerance. We next determined whether Ad vector-induced defects in insulin signaling in liver could alter systemic glucose homeostasis. Body weight was not different between the Ad vector-infected and control rats (Fig. 7A). The fasting blood glucose level was slightly, but significantly, elevated in rats with Ad vector infection (Fig. 7B). Compared with the PBS-injected rats, animals treated with Ad vector became glucose intolerant at 6 days postinjection as determined by glucose tolerance tests (Fig. 7B). Impaired glucose tolerance by Ad vector injection was not apparent by 13 days, indicating that the effect of Ad vector on whole body glucose metabolism had returned to normal by that time (Fig. 7C).

**DISCUSSION**

Recombinant Ad vectors have been proven to be highly promising as gene delivery vehicles (41, 44). However, the use of Ad-based vectors is limited by inflammatory responses to the vector (31). Despite increased efforts to better understand Ad infection (18, 26, 29, 33, 34), little is known about how it affects cellular and whole body metabolism, insulin signaling, and glucose homeostasis. Characterizing the effects of Ad vector on insulin action can provide insights for discovering virus infection-induced insulin resistance and is critical for understanding the metabolic side effects of Ad-mediated gene therapy and developing strategies to improve the application of Ad vectors in clinical or basic research.

In the present study, we demonstrated that systemic administration of Ad vectors impaired hepatic insulin signaling and glucose metabolism. Several nonreplicative Ad vectors, Ad5-LacZ, Ad5-nt-LacZ, and Ad5-GFP (not shown), were studied,
and they consistently resulted in a decrease of hepatic IR, IRS-1, and IRS-2 following their administration. Ad vectors are mostly sequestered by the liver after systemic administration, where the Kupffer cells can be activated (34, 44). Unlike in liver, insulin signaling in skeletal muscle and adipose tissue was not impaired by Ad vector administration. Previous studies (30, 34), as well as the present study, indicate that muscle and fat are not affected by Ad serotype 5 (Ad5) after systemic administration, and this lack of infection by Ad vectors likely accounts for the lack of effects on insulin signaling in these two tissues.

Liver is the major organ for glucose production and storage and plays a central role in maintaining glucose homeostasis (25). Although within the normal range, fasting blood glucose was elevated after Ad vector administration compared with uninjected rats. Systemic glucose homeostasis was impaired by Ad vector infection as determined by glucose tolerance tests, likely arising from the failure of insulin to suppress hepatic glucose production following the glucose load. Our data also indicate that, once the protein level started to return (IRS-1) or returned (IR, IRS-2) to normal by 13 days, systemic glucose homeostasis also normalized.

The potential roles of viral infection in the pathogenesis of insulin resistance and type 2 diabetes have been implicated in recent years (7, 11, 14). Viruses, including hepatitis C and B viruses, have been linked to hepatic insulin resistance (14, 20). Similar to Ad vector, hepatitis C virus mainly targets the liver, and the protein levels of IRS-1 and IRS-2 are reduced in liver from patients with hepatitis C infection and hepatitis C virus core transgenic mice (11). In addition, several studies suggested a role of TNFα in hepatitis C virus-associated insulin resistance (12, 20). Thus, similarities between hepatitis C infection and what we have found with Ad vector suggest that they may both result in a state of insulin resistance, possibly through similar mechanisms. Therefore, exploring the mechanisms underlying Ad vector-induced defects in insulin signaling may provide additional insights for understanding the pathogenesis of viral infection-related insulin resistance.

One possible mechanism is increased degradation of hepatic IR, IRS-1, and IRS-2. Systemic administration of Ad vector activates Kupffer cells, resulting in production of inflammatory factors and inflammation in liver (28, 33). TNFα inhibits IR activity and promotes ubiquitination and degradation of IRS proteins through multiple mechanisms, including upregulating expression of the suppressor of cytokine signaling (SOCS) proteins (15, 23, 32, 43) and promoting IRS serine phosphorylation (9, 10). Increased IRS-1 Ser307 phosphorylation was observed 4 days after injection of Ad5-nt-LacZ, and this increase of IRS-1 Ser307 phosphorylation may help target IRS-1 for degradation.

In the present study, the decreased protein levels of IR and IRS-1 by Ad vector infection were completely or partially reversed by TNFα inhibitor expression, indicating a role of TNFα in Ad vector-induced defects in liver insulin signaling and maybe also accounting for the induction of serine phosphorylation of IRS-1. However, TNFα inhibition only partially prevented the Ad vector-induced decreases in IRS-1. One possible explanation is that the TNF inhibitor takes several days to be maximally expressed, and the negative effects of Ad vector infection already occurred before significant expression of the TNF inhibitor. Alternatively, other factors in addition to TNFα, such as IL-6, may cause the reductions after Ad vector infection.

Kupffer cells are a source of TNFα following Ad vector administration, and Kupffer cell depletion decreases production of TNFα (17, 45). Consistent with previous studies by others (8), GdCl3 treatment dramatically reduced the number of Kupffer cells in rat liver. Kupffer cell depletion by GdCl3 significantly attenuated Ad vector-induced reduction of insulin signaling molecules, suggesting involvement of Kupffer cell activation in Ad vector infection-impaired insulin signaling. In addition to increased degradation, the observed decreases in IR, IRS-1, and IRS-2 proteins by Ad vector could also occur by specific inhibition of mRNA or protein synthesis, and analysis of mRNA levels of these three molecules will provide additional insight into this situation.
In addition to insulin resistance, viral infection can result in other metabolic abnormalities, such as obesity and liver steatosis. Ad vector infection has been reported to increase adiposity and obesity in mice (36). Patients with chronic hepatitis C or B have an increased risk for developing liver steatosis (2, 14, 42). Studies using cell culture and animal models demonstrate a role of hepatitis C virus core protein in the pathogenesis of liver steatosis (3, 27). On the basis of the similarities between Ad vector and hepatitis C infection, and the strong relationship between insulin resistance and lipid metabolism, Ad vectors may have the potential to promote the development of liver steatosis, which remains to be determined.

Although Ad vectors have limitations due to their immunogenicity, Ad vector delivery systems comprise ~25% of all past and current human gene therapy clinical trials (www.wiley. co.uk/genmedclinical). Inflammatory responses to Ad vector infection occur in humans, rats, and mice (29, 34, 41). Thus, the defects in insulin action may also occur in human patients receiving Ad vector-based gene therapy, and Ad-based gene therapy could be a risk factor for insulin resistance and metabolic disorders.

E1/E3-deleted Ad vectors are also widely used in basic research, including research in the area of insulin signaling and diabetes (5, 30). Our findings suggest that the Ad vector itself can affect the protein levels of IR, IRS-1, and IRS-2 in liver. Thus, effects of the viral vector may confound studies using these vectors in insulin signal transduction and insulin resistance studies, especially if the liver is studied.

The development of modified Ad vectors, deleting a majority of the viral genome to minimize adaptive immune response, does not prevent activation of the early innate immune response, including the acute release of cytokines and chemokines, and tissue inflammatory injury (19, 29). Our studies using UV-inactivated virus also suggests that the innate immune response by inactivated viral particles can still partially alter insulin signaling molecule expression in liver. Thus, strategies targeting the innate immunity of Ad or other virus vectors are also important for the future development of safer vectors for use in human gene therapy.

In summary, we demonstrate that systemic administration of recombinant adenovirus vectors can alter insulin signaling in liver and impair systemic glucose homeostasis. Thus, effects of adenovirus on insulin action and glucose metabolism need to be considered when adenovirus vectors are used in research or human gene therapy and may be more broadly applicable to other viral agents.

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DISCLOSURES

No conflicts of interest are reported by the authors.

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