Research article

E4orf1, an Adeno-viral protein, attenuates renal lipid accumulation in high fat fed mice: A novel approach to reduce a key risk factor for chronic kidney disease

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ABSTRACT

Obesity and hyperlipidemia are independent risk factors of chronic kidney disease (CKD). In mice, diet induced obesity accelerates lipogenesis, lipid accumulation, and injury in kidneys. Expression of adenoviral protein, E4orf1, improves glucose clearance and reduces endogenous insulin secretion to glucose challenge in mice. Therefore, in this pilot study, we examined, if enhanced glycemic control in HFD fed E4orf1 transgenic (E4orf1-Tg) mice, will reduce renal lipogenesis and lipid accumulation. In two separate experiments, E4orf1-Tg mice were fed 60% (kcal) high-fat diet (HFD) supplemented with doxycycline for 10-weeks or 20-weeks along with wild-type (C57BL6/J) or E4orf1-non-transgenic (E4orf1-non-Tg) control mice, respectively. Protein expression of Fatty Acid Synthase (FAS) and Acetyl-CoA Carboxylase (ACC), accumulation of triglyceride (TG) along with mRNA levels of lipid metabolism and injury markers were determined in kidneys. Renal expression of FAS and ACC, and TG content was significantly reduced in E4orf1-Tg mice compared to controls. E4orf1-Tg mice show significant increase in genes involved in mitochondrial fatty acid oxidation and oxidative stress compared to wild-type mice after 10-weeks of HFD. However, mice exposed to 20-weeks of HFD, show no difference in gene expression. E4orf1 expression reduces lipid synthesis and accumulation in kidneys despite HFD, which may be due to attenuation of hyperinsulinemia by E4orf1.

1. Introduction

High dietary fat is a known risk factor in the development of metabolic syndrome (MetS) both in humans and in rodent models [1, 2]. MetS is defined as a cluster of insulin resistance, type 2 diabetes, impaired glucose levels along with additional risk factors from obesity, non-alcoholic fatty liver disease (NAFLD), dyslipidemia, hypertension, and microalbuminuria [3]. Even though MetS is associated with chronic kidney disease (CKD) [4, 5], however, the pathophysiological mechanism is not well understood [6]. Several mechanisms have been proposed based on hyperglycemia, hyperinsulinemia/insulin resistance, dyslipidemia, ectopic fat accumulation, lipotoxicity, and renal injury [7]. Mice fed a high-fat diet (HFD) show hyperglycemia as well as altered lipid metabolism, excessive lipid accumulation and injury in kidneys [8, 9].

Local lipid metabolism and accumulation in different tissues is controlled by many enzymes and transcription factors like Acetyl-CoA Carboxylase (ACC), Fatty Acid Synthase (FAS), Carnitine Palmitoyltransferase (CPT), Sterol Regulatory Element–Binding Proteins (SREBPs), Carbohydrate-Response Element Binding Protein (ChREBP), Peroxisome Proliferator-Activated Receptors (PPARs), Cluster Differentiation 36 (CD 36), Acyl-CoA Thioesterase (ACOT) and β-Hydroxy β-MethylGlutaryl-CoA (HMGCoa) synthase/reductase [6]. Level, expression or function of these molecules are altered in MetS associated renal lipid accumulation and CKD [6, 8, 9, 10]. ACC and FAS are two key enzymes for FA synthesis [10], CPT is involved in intracellular FA export from the cytosol to mitochondria [8, 10], ACOT catalyzes FA oxidation [11], whereas HMGCoaS gives protection against oxidative stress [12]. SREBP1c (member of SREBPs) [9], ChREBPs [13] and PPAR- α, β, γ [14] are all well-known transcription factors that regulate lipid metabolism. Although hyperglycemia, hyperinsulinemia/insulin resistance, and dyslipidemia are implicated in the alteration of these enzymes and transcription factors during HFD-induced impaired renal lipid metabolism, the complete pathway is not yet known.

In vitro and in vivo studies have collectively shown that early gene 4 open reading frame-1 (E4orf1), a 17 KDa, protein of the human adenovirus-36 (Ad36), acts as an anti-hyperglycemic agent [15, 16, 17].

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In vitro, E4orf1 mediates the insulin signaling pathway by increasing the expression of phosphoinositide 3-kinase (PI3K) and PKB/akt (protein kinase B) during insulin resistance, and increases cellular translocation of glucose transporter 4 (GLUT 4) to facilitate glucose uptake [18]. Similarly, tissue specific or systemic in vivo expression of E4orf1 in mice shows improved glycemic control in an insulin independent manner despite exposure to high fat diet [19, 20, 21]. The insulin-independent glucose disposal by E4orf1 reduces the requirement of endogenous insulin for glucose disposal, which in turn reduces hyperinsulinemia [15, 20].

In HepG2 liver cells, E4orf1 expression reduces glucose output, de-novo lipogenesis, but increases complete oxidation of FA, and promotes lipid export [22]. Further, in mice, reducing endogenous insulin by E4orf1 protects against hepatic lipid accumulation [15, 21]. As E4orf1 reduces endogenous insulin requirement, improves glycemic control, and protects against HFD-induced hepatic steatosis, we asked the question, “Whether E4orf1 will prevent or protect against HFD-induced lipid accumulation in the kidney?” In this proof of concept study, we determined if improvement in glycemic control with reduced endogenous insulin output in HFD-fed E4orf1-Transgenic (E4orf1-Tg) mice will reduce renal lipogenesis and lipid accumulation. We analyzed genes and proteins specifically involved in lipid synthesis, oxidation, and transport in kidneys and examined the role of E4orf1 in preventing HFD-induced renal fat accumulation in two different cohorts of mice.

2. Materials and methods

2.1. Animal models

All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of Texas Tech University (TTU). Wild type C57BL/6J and E4orf1-Tg and E4orf1-non-transgenic (E4orf1-non-Tg) mice of C57BL/6J background were obtained from breeding colony at TTU. These mice inductively express the human Ad36-E4orf1 protein in the adipose tissue upon doxycycline (dox) feeding. The E4orf1-Tg mice generation and characterization have been previously described [20]. In brief, E4orf1 cDNA was cloned using pTRE vector, which has a CMV promoter with 7 tandem repeats of the tetracycline-responsive element (TRE) after it to control E4orf1 expression. A purified DNA fragment of 5.2 Kbp was inserted into C57BL/6J mice and used to generate TRE-E4orf1 offspring. Transgene positive mice were distinguished by genotyping using following primers: 5'-GGGACATGAAACAGCTGCAATG and 5'-AATTACCTCCTCCAGCGGCGG, and then cross bred with adiponectin-rtTA (AdipoQ-rtTA) mice. Adiponectin-rtTA mice have rTA (an essential transcription factor to active TRE and make the promoter functional) under the control of the adiponectin promoter [20]. Transgene positive offspring was distinguished by previously mentioned primer set and used for further experiments with dox administration. All mice were placed in group housing (≤5 per cage) for a 12-hour light/12-hour dark cycle. They had access to an ad-libitum diet and water throughout the study. Only age-matched male and female mice were used for experiment 1. Weekly body weights were measured for all mice. We did not measure food intake as previous study confirms no changes in food intake or metabolic activity for E4orf1-Tg mice [20].

2.2. Experiment 1

Twenty-nine-week-old, male E4orf1-Tg mice (n = 5; as determined by genotyping) along with wild type male (n = 5; C57BL/6J) mice were used for this experiment. The detailed experimental design has been previously described [15]. In this experiment, mice were fed rodent chow diet supplemented with 600 mg/Kg doxycycline (chow-dox) (16% Kcal fat, Research Diets) diet for 6 weeks followed by 10 weeks of high fat-doxycycline (HF-dox) (10% Kcal, Research Diet). After 16 weeks, mice were sacrificed, and tissues collected for further analysis. Expression of E4orf1 protein in adipose tissue was confirmed by western blot analysis for all E4orf1-Tg mice. Among them, around 40% were positive for E4orf1 protein expression [15] and tissues from these mice were considered for final analysis.

2.3. Experiment 2

Fifty weeks old age-matched male and female E4orf1-Tg mice (n = 5; as determined by genotyping) along with E4orf1-non-Tg mice (n = 5; as determined by genotyping) were exposed to HF-dox diet for 20 weeks ad-libitum. Following 20 weeks of HF-dox feeding, mice were sacrificed, and tissues were collected for further analysis. Expression of E4orf1 protein in the adipose tissue was confirmed by western blot analysis in all E4orf1-Tg mice.

2.4. Western blot analysis

Kidneys from mice (from Experiments 1 and 2) were homogenized using RIPA lysis buffer supplemented with protease inhibitor (Sigma Aldrich, cat. # P8340). Protein concentration was determined by BCA assay (BCA kit, Thermo Fisher, cat. # 23225). SDS-PAGE was performed with 20 μg protein loaded onto a 7% polyacrylamide gel and size separated proteins were transferred onto a nitrocellulose membrane (for proteins >200 kDa) or PVDF membrane (for proteins< 200KDa). Primary antibodies for ACC (Cell signaling, 3662S), FAS, (BD Bio, cat. # 610963), and β-actin (cell signaling, 3700) were used at a 1:1000 dilution. Appropriate (rabbit, goat, or mouse) horse reddish peroxidase (HRP) was used as secondary antibody and Clarity western ECL substrate (Biorad, cat. # 170-5061) was used to visualize protein bands on the membranes. Finally, the protein bands were captured using a BioRad ChemiDoc™ imager and analyzed using the NIH ImageJ software.

2.5. RNA extraction and cDNA preparation

RNeasy® Plus Universal Mini Kit (cat. # 73404) was used to extract total RNA from the kidneys. Tissue samples of each mouse at the time of sacrifice was collected in 2 ml tubes containing Invitrogen™ RNA later™ Stabilization Solution (Fisher Scientific cat # AM7021) and dissolved in QIAzol® reagent (cat. # 79306). All the tissues were homogenized with TissueLyser LT for 5 min at 50 Hz and RNA was extracted according to the manufacturer’s protocol. Complementary DNA (cDNA) was synthesized using the Maxima H Minus First Strand Complementary DNA Synthesis Kit (Thermo Fisher Scientific, cat. #K1681) with 4μg of RNA.

2.6. Quantitative real-time polymerase chain reaction (qRT-PCR)

Genes expression associated with renal lipid synthesis and oxidation was determined by qRT-PCR. PCR reactions were carried out in 96-well plates with a final volume of 20 μl in each well. 25 or50 ng of cDNA along with 450 nM forward and reverse primers, and 10 μl of 1X SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad Laboratories, cat. # 17–5271) was used as a PCR mix and the reactions were detected using Bio-Rad CFX RT-PCR detection system. Primers were designed using Sigma Aldrich Oligo Architect software. A list of primers has been presented in Table 1. Mouse TATA-binding protein (TBP), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β2 Microglobulin (B2m) and β-actin genes were tested as reference (housekeeping) genes in kidneys for this study. Among them, B2m or β-actin were mostly conserved and used as the reference gene for experiment 1 and 2, respectively. All the reactions were performed in duplicates and averages were used to calculate fold changes in mRNA expression (2^−ΔΔCT method).
2.7. Renal TG concentration measurement

Renal TG content (mg/g) was determined using the Cayman kit (cat. # 10010303). Approximately 100 mg of tissue sample from each mouse was weighed and homogenized in 1X RIPA buffer with 1X protease inhibitor using Tissulyser LT (Qiazen, cat. # 69980). Further analysis was carried out according to the manufacturers protocol (Cayman kit, cat. # 10010303). TG concentration was normalized to the total tissue (g) as well as total protein (g).

2.8. Data analysis

All results are presented as mean ± standard error of the mean. Comparison between two groups was performed using Welch’s t-test, assuming unequal variance in the values for each group.

3. Results

3.1. E4orf1 expression does not alter kidney tissue weights during high fat feeding

In both experiments, kidneys from the mice were collected during sacrifice and immediately weighed. Kidney tissue weights for E4orf1-Tg and control mice were not significantly different in either of the experiments (Figure 1 a and b). Moreover, there was no difference in kidney weights normalized to total body weight in both experiments (Figure 1 c and d). Total body weight differed between E4orf1-Tg and wild type mice after 6 weeks of chow-dox feeding, while the body weights were not statistically significant after 10 weeks of HF-dox feeding (experiment 1) [15]. Akheruzzaman, et.al., showed that induction of E4orf1 slowed down weight gain in E4orf1-Tg mice compared with wild type mice. In experiment 2, E4orf1 expression was induced during HF-dox diet for 20 weeks and no difference in total body weight was observed between E4orf1-Tg and E4orf1-non-Tg mice (Mostoñejad and Hegde unpublished observations). Furthermore, in both experiments improved glucose clearance along with reduced endogenous insulin level was found during glucose tolerance test (GTT) in E4orf1-Tg compared to wild type [15] and in E4orf1-Tg mice compared to E4orf1-non-Tg mice (Mostoñejad and Hegde unpublished observations).

3.2. E4orf1 significantly reduces expression of lipogenic proteins in kidneys

In experiment 1, western blot analysis showed a significant decrease in protein expression for both Fasn (p < 0.007) and Acc (p < 0.05) genes in kidneys from E4orf1-Tg mice compared with wild type mice (Figure 2).
Finally, expression of renal mitochondrial in FAS (p \(<\) 0.05) and ACC (p \(<\) 0.03) protein expression in E4orf1-Tg mice compared with E4orf1-non-Tg mice (Figure 2 d, e, and f) [23].

3.3. E4orf1 significantly reduces renal TG concentration

In experiments 1 and 2, renal TG concentration was significantly lower in E4orf1-Tg mice compared to wild type (experiment 1) or E4orf1-non-Tg mice (experiment 2) respectively (Figure 3) [23]. In experiment 1, following 10 weeks of HFD feeding, the TG amount normalized to tissue weight (Figure 3 a) was significantly lower (p \(<\) 0.03) in E4orf1-Tg mice compared with wild type mice. TG amount did not differ between the two groups after normalization to total protein (Figure 3 b). However, in experiment 2, despite 20 weeks of HFD feeding, TG amount normalized for both the tissue weight (Figure 3 c; p < 0.006) and total protein (Figure 3 d; p < 0.008) showed a statistically significant reduction in E4orf1-Tg mice compared to E4orf1-non-Tg mice.

3.4. E4orf1 expression alters mRNA levels of genes involved in renal lipid metabolism

To determine the changes in expression of genes involved in renal lipid metabolism in the presence of E4orf1 expression, we performed qRT-PCR. mRNA samples extracted from kidney tissues of mice exposed to 10 weeks (Experiment 1) and 20 weeks of HFD (Experiment 2) were used to determine the expression of selective genes involved in de novo lipogenesis, FA uptake, fat oxidation, and oxidative stress.

In Experiment 1, mRNA expression of genes involved in renal de novo lipogenesis (Fasn, Acc1, Acc2, and Chrebp) did not differ significantly between E4orf1-Tg and wild type mice after 10 weeks of HFD feeding (Figure 4 a). The expression of Cd36 gene, which is involved in FA uptake, did not differ between groups (Figure 4 b). Interestingly, for genes involved in FA oxidation, Cpt1a did not show any significant difference, while Pparγ though not significant, shows a 2-fold higher expression in E4orf1-Tg mice compared with wild type mice (Figure 4 b). However, Acac2 involved in mitochondrial FA oxidation showed 3.5-fold increase (p \(<\) 0.03) in E4orf1-Tg mice compared with wild type mice (Figure 4 b). Finally, expression of renal mitochondrial HmgCoAs was 6-fold higher in E4orf1-Tg mice (p < 0.06) compared with wild type control mice (Figure 4 b) suggesting protection against enhanced oxidative stress. Fold changes in mRNA levels were normalized using B2m as a housekeeping gene in experiment 1.

In Experiment 2, following 20 weeks of HFD, we did not observe any differences in mRNA expression for genes involved in renal de novo lipogenesis, FA uptake, its oxidation and oxidative stress in E4orf1-Tg mice compared to E4orf1-non-Tg mice (Figure 4 d and e). In experiment 2, β-actin was used as a housekeeping gene to normalize fold changes in mRNA levels. Moreover, there were no significant changes in mRNA levels for genes involved in renal inflammation for both experiments (Figure 4 c and f).

4. Discussion

This proof of concept study demonstrated that expression of adenviral protein, E4orf1 in adipose tissue attenuates HFD-induced renal lipid accumulation compared with wild type or E4orf1-non-Tg mice. In high fat fed mice, E4orf1 expression improves glycemic control with reduced endogenous insulin secretion and prevents lipid accumulation in the liver [15, 17, 19]. Here we present role of adipose tissue specific E4orf1 expression in attenuation of renal lipid metabolism despite HFD.

Several rodent studies have shown the association between HFD, hyperinsulinemia, hyperglycemia and renal lipid accumulation [6, 24, 25, 26]. Therefore, we determined a) renal lipogenesis in E4orf1-Tg mice during HFD feeding and b) if the improvement in hyperglycemia and hyperinsulinemia observed with E4orf1 expression will prevent renal lipid accumulation. Transgenic mice inductively expressing E4orf1 in the adipose tissue and exposed to HFD for 10 weeks or 20 weeks, show significantly lower expression of the lipogenic proteins, FAS and ACC in kidneys compared to wild type or E4orf1-non-Tg mice, respectively. These observations suggest that the improvement in glycemic control independent of endogenous insulin secretion by the E4orf1 expressing mice [15] (Mostofinejad and Hegde unpublished observations), protects against hyperinsulinenia/insulin resistance and subsequent renal lipid accumulation.

Despite 10 and 20 weeks of HFD, E4orf1-Tg mice show a significant reduction in renal TG content compared to wild type and E4orf1-non-Tg mice, respectively. Lower TG amount in kidney is in agreement with

![Figure 2](image-url). E4orf1 expression modulates lipid synthesis in kidney. In mice, E4orf1 expression significantly reduced kidney protein expression of genes involved in lipid biosynthesis despite HFD feeding. Western blot analysis of FAS and ACC from mice exposed to high fat diet for 10 weeks (a) and 20 weeks (d). Protein expression normalized to β-actin in mice exposed to 10 weeks and 20 weeks high fat feeding is shown for FAS (b and e) and ACC (c and f) respectively. Data are presented as mean ± SEM and n = 5 mice per group. Welch’s t test: *p < 0.05 and **p < 0.01.
significant lower protein expression of lipid synthesis genes, Fasn and Acc, which are downstream targets of Srebp1c [14]. In mice, expression of SREBP1c and ChREBP proteins are increased in kidneys during HFD [9].

To determine the putative mechanisms of increased TG accumulation in the kidney, we examined the mRNA expression of genes that play a role in regulating FA metabolism. Contrary to protein expression, after 10 weeks of HFD feeding, E4orf1-Tg and wild type mice did not show any significant difference with mRNA expression of genes involved in renal lipogenesis (Fasn, Acc1, Acc2, Srebp1c, Chrebp) or FA uptake (Cd 36). Upregulation of Pparγ and Acot2 suggests an enhanced renal lipid oxidation in E4orf1-Tg mice compared to wild type, whereas, upregulation of mitochondrial HmgCoAS indicates possible protection from oxidative stress. Collectively, in mice exposed to 10 weeks of HFD, these data suggest that E4orf1 expression improves glycemic control [15] and prevents lipid accumulation in the kidneys due to attenuated lipogenesis, FA uptake, oxidative stress and increased fat oxidation.

However, E4orf1 expressing mice exposed to 20 weeks of HFD do not show any change in genes involved in renal lipogenesis, FA uptake and oxidation along with oxidative stress in E4orf1-Tg mice compared to E4orf1-non-Tg mice. This discrepancy could be due to the difference in age (35-week vs 50-week old) at the time of starting HFD feeding and the duration of HFD feeding (10-week vs 20-week) between the two cohorts of mice. Differences in body weight could also causes these changes. In experiment 1, at 35 weeks of age, E4orf1-Tg mice had significantly higher body weight and fat mass at baseline compared to age matched wild type mice on chow diet [15]. Similarly, in experiment 2, at 50 weeks of age, though not significantly different, E4orf1-non-Tg mice had higher body weight compared with E4orf1-Tg mice, 47.2g–41.2g respectively (Mostofinejad and Hegde unpublished observations). In C57BL/6J mice there is a progressive increase in age-related lipid accumulation with corresponding increase in renal TG [27]. Additionally, diet-induced obesity in C57BL/6J causes increased renal lipid accumulation by activating the SREBP1c pathway [9]. Depending on the duration of HFD feeding there is an upregulation in expression of genes involved in lipogenesis, which gets saturated in the longer term [8, 9]. Furthermore, in experiment 1, E4orf1 expression was induced for 6 weeks with a corresponding increase in renal TG [27]. Additionally, diet-induced obesity in C57BL/6J causes increased renal lipid accumulation by activating the SREBP1c pathway [9]. Depending on the duration of HFD feeding there is an upregulation in expression of genes involved in lipogenesis, which gets saturated in the longer term. In experiment 2, following 20 weeks of high fat feeding, (d) shows changes in mRNA expression for genes involved in renal de novo lipogenesis (Fasn, Acc1, Acc2, Srebp1c, Chrebp) and (e) for changes in genes involved in FA uptake (Cd36), fat oxidation (Pparγ, Cpt1a and Acot2) and TG metabolism (HmgCoAS). In Experiment 2, following 20 weeks of high fat feeding, (a) shows changes in mRNA expression for genes involved in renal de novo lipogenesis (Fasn, Acc1, Acc2, Srebp1c, Chrebp) and (b) for changes in genes involved in FA uptake (Cd36), fat oxidation (Pparγ, Cpt1a and Acot2) and TG metabolism (HmgCoAS). (e) and (f) shows inflammatory markers (Mcp1, Tnfa and Il6) in kidneys for experiment and experiment 2 respectively. Data are presented as mean ± SEM and n = 4 mice per group. Welch’s t-test: *p < 0.05.

Rodent studies have demonstrated an association between HFD and renal lipid accumulation, along with hyperglycemia, hyperinsulinemia, insulin resistance and obesity as the modulating factors [6, 25, 26], but the applicability of these findings to human kidney disease remains to be established. This is the first report for the role of adipose tissue specific E4orf1 expression in regulating renal lipid metabolism despite HFD. A possible mechanism could be E4orf1-mediated improved glycemic control without increasing endogenous insulin secretion or hyperinsulinemia. Additional investigations are warranted to determine molecular mechanism of E4orf1-mediated adipose tissue modulation of renal lipid accumulation and protection from HFD-induced renal injury.
Declarations

Author contribution statement

V. Hedge: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

R. Afruza: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Md. Akheruzzaman: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

N.V. Dhurandhar: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Competing interest statement

The authors declare the following conflict of interests: N.V. Dhurandar has received several United States and international patents that protect intellectual property about the use of adenoviruses and its proteins in obesity, diabetes, and related areas.

Additional information

No additional information is available for this paper.

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