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Research paper

An adenovirus-derived protein: A novel candidate for anti-diabetic drug development

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ABSTRACT

Aims: Exposure to human adenovirus Ad36 is causatively and correlatively linked with better glycemic control in animals and humans, respectively. Although the anti-hyperglycemic property of Ad36 may offer some therapeutic potential, it is impractical to use an infectious agent for therapeutic benefit. Cell-based studies identified that Ad36 enhances cellular glucose disposal via its E4orf1 protein. Ability to improve glycemic control in vivo is a critical prerequisite for further investigating the therapeutic potential of E4orf1. Therefore, the aim of this study was to determine the ability of E4orf1 to improve glycemic control independent of insulin despite high fat diet.

Materials & Methods: 8–9wk old male C57BL/6J mice fed a high-fat diet (60% kcal) were injected with a retrovirus plasmid expressing E4orf1, or a null vector (Control). Glycemic control was determined by glucose and insulin tolerance test. Islet cell size, amount of insulin and glucagon were determined in formalin-fixed pancreas. Rat insulinoma cell line (832/13) was infected with E4orf1 or control to determine changes in glucose stimulated insulin secretion. Protein from flash frozen adipose tissue depots, liver and muscle was used to determine molecular signaling by western blotting.

Results: In multiple experiments, retrovirus-mediated E4orf1 expression in C57BL/6J mice significantly and reproducibly improved glucose excursion following a glucose load despite a high fat diet (60% energy). Importantly, E4orf1 improved glucose clearance without increasing insulin sensitivity, production or secretion, underscoring its insulin-independent effect. E4orf1 modulated molecular signaling in mice tissue, which included greater protein abundance of adiponectin, p-AKT and Glucose transporter Glu4.

Conclusions: This study provides the proof of concept for translational development of E4orf1 as a potential anti-diabetic agent. High fat intake and impaired insulin signaling are often associated with obesity, diabetes and insulin resistance. Hence, the ability of E4orf1 to improve glycemic control despite high fat diet and independent of insulin, is particularly attractive.

1. Introduction

Although about a dozen classes of anti-diabetes drugs are available, considerable need for better anti-diabetes drugs persists [1]. For example, high fat intake or impaired insulin signaling are often associated with diabetes. Yet, the current anti-diabetes drugs are insulin sensitizers, insulin mimetic or secretagogues, requiring insulin signaling for optimal benefits. Therefore, drugs that improve diabetes independent of dietary fat intake and insulin action may offer a significant advantage [2]. Here we describe such potential of the E4orf1 protein of human adenovirus Ad36, which may be translated to develop new anti-diabetic agents.

Microbes are a somewhat unconventional yet important source to develop therapeutic agents and strategies [3]. In animal models, Ad36 increases adiposity, but enhances glycemic control and reduces hepatic lipid accumulation, despite high fat diet and without recruiting the proximal insulin signaling [4–7]. Ad36 appears to enhance systemic glycemic control by promoting glucose uptake by adipose tissue and skeletal muscle, and by reducing hepatic glucose

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output and hepatic steatosis [8,9]. In rhesus monkeys, natural exposure to Ad36 is linked with a reduction in fasting glucose levels [10]. Interestingly, as cross-sectional and prospective associations, the key findings from Ad36-infected animals are mirrored in humans who are naturally exposed to Ad36 infection [4,11–22]. In humans, natural exposure to Ad36 is associated with obesity and fat gain, yet lower liver lipids and better glycemic control [4,11–22]. Natural Ad36 infection has also been shown to be associated with lower occurrence of type 2 diabetes with increased insulin sensitivity [23].

The potential of Ad36 to improve glycemic control is highly attractive for developing an effective anti-diabetic agent. Nonetheless, it is impractical to use a viral infection to improve diabetes. Instead, in vitro experiments identified that the E4orf1 protein, a 125 amino acid peptide [24], increases cellular glucose uptake in preadipocytes, adipocytes, and myoblasts, and reduces glucose output from hepatocytes [25,26]. These studies showed that E4orf1 bypassed the IRS mediated proximal insulin signaling and improved cellular glucose by up-regulating the distal insulin signaling involving phosphatidyl insositol 3-kinase (PI3K), Akt and glucose transporter 4 (Glut4), via Ras activation [27]. These observations collectively showed that unlike the current anti-diabetic drugs, E4orf1 did not function as an insulin sensitizer, mimetic or secretagogue, but exhibited an insulin sparing effect [27]. This finding provided a single protein instead of an infectious agent, as a candidate to further investigate the anti-diabetic potential of Ad36 in vivo. Either the E4orf1 protein itself, or its analogs may be useful for developing therapeutic agent(s), provided, the protein improves glycemic control in vivo.

A proof of concept that E4orf1 protein enhances glycemic control in vivo is essential for further translating therapeutic properties of E4orf1. Hence, in multiple experiments, with the help of a retrovirus vector (pBabe), we expressed Ad36 *E4orf1* gene in high fat fed male C57BL/6J mice to determine the effect on glucose and insulin response and related cell signaling, and to assess the duration and reproducibility of the effect. As described below, the results demonstrated that E4orf1 is capable of improving glycemic control in vivo. This in vivo validation should stimulate further research towards developing E4orf1-based therapeutic agents.

2. Materials & Methods

2.1. Animal experiments

Institutional Animal Care and Use Committee (IACUC) of the Pennington Biomedical Research Center approved the protocols for animal studies. Mice were purchased from The Jackson Laboratories (Bar Harbour, Maine, USA) and placed on a 12 h light–dark cycle at 25 °C and housed in micro-isolator cages under Biosafety level-2 containment, with ad libitum access to food and water. End of study sacrifice was conducted by CO_2 asphyxiation followed by cervical dislocation. Based on a previous study [4] power analysis showed that a total of 6–9 mice were required in this parallel design study, at 90% probability to detect a treatment difference at a two-sided significance at 0.05.

Experiment 1: Does E4orf1 enhance blood glucose excursion in high fat fed mice?

Six-week old C57Bl/6J male mice on rodent chow (Purina Lab Diet 5001) were allowed 1-week of acclimatization following which total body fat was determined by Bruker Minispec mq10-NMR (Nuclear Magnetic Resonance) analyzer. Baseline GTT was performed as described under methods, to determine blood glucose clearance. The animals were then placed on a high fat (HF, 60% kcal) diet (Research Diets Inc. D12492i) for 2 weeks and GTT performed to determine HF diet induced hyperglycemia. Mice were divided into two groups (n = 9 each; Control or E4orf1) matched for body weight and inoculated with 300 μL pBabe-puro (Control) or pBabe-E4orf1 (10^8 copies of E4orf1) retrovirus as a combination of intra-peritoneal (i.p.), intra muscular (I.M.) and subcutaneous injections. GTT was performed 1 week post-infection (p.i.).

Experiment 2: Does E4orf1 transiently but reproducibly enhance glucose excursion?

Nine week old C57Bl/6J male mice on HF diet since 6 week of age were weight matched, divided into two groups and inoculated with pBabe-puro (Control; n = 6) or pBabe-E4orf1 (E4orf1; n = 6) retrovirus as described in Experiment 1. GTT was performed 1, 2, 3 and 4 week p.i. Mice were re-inoculated on week 7 p.i. and GTT performed 4 and 7 days post re-inoculation.

Experiment 3: Does a booster dose of E4orf1 extend the duration of enhancement in glucose clearance?

Eight week old C57Bl/6J male mice on HF diet since 6 wk of age, after 1 week of acclimatization and total body fat determination were weight matched into two groups. At 9 week of age the mice were inoculated with pBabe-puro (Control; n = 6) or pBabe-E4orf1 (E4orf1; n = 6) retrovirus as described in Experiment 1. GTT was performed 1 week p.i. and mice were re-inoculated at 10 week of age and GTT performed 1 week post re-inoculation to determine booster dose effect on blood glucose clearance. GTT was also performed 2 week post re-inoculation and 1 week post second booster inoculation.

Experiment 4: Does longer duration of high fat diet delay the improvement in GTT induced by E4orf1?

Eighteen C57Bl/6J male mice on HF diet since 6 week of age were weight matched, divided into three groups and total body fat content was determined. At 14 week of age the mice were inoculated with pBabe-puro (Control; n = 6) or pBabe-E4orf1 (E4orf1 300 μL; n = 6) or pBabe-E4orf1 (E4orf1 600 μL; n = 6) retrovirus as described in Experiment 1. GTT was performed 1, 2 and 3 week p.i. following which the mice were re-inoculated with a booster dose and GTT performed 4 days post re-inoculation.

Experiment 5: Does E4orf1 improve glucose clearance without increasing insulin sensitivity, production or secretion?

Nine week old C57Bl/6J male mice on HF diet since 6 week of age were weight matched, divided into two groups and inoculated with pBabe-puro (Control; n = 3) or pBabe-E4orf1 (E4orf1; n = 3) retrovirus as described in Experiment 1. Insulin tolerance test (ITT) was performed 1 and 2 week p.i. At the time of sacrifice (described in Experiment 6), trunk blood was collected and serum separated from fasted and non -fasted control (n = 3) and E4orf1 (n = 3) mice. Serum insulin was measured using insulin kit (Rat/Mouse Insulin ELISA, #EZRMI-13K, Millipore). Formalin fixed pancreas from control (n = 3) and E4orf1 (n = 3) were sectioned and beta cell mass was determined by selecting insulin expressing beta cell areas. These areas were then combined within a slice and the total area of insulin expressing cells was divided by the total tissue area on the slide and multiplied by 100. This gave the percent pancreas 2D section covered by beta-cells. The sectioned pancreata from multiple mice were also stained for insulin and glucagon to measure intensity between beta and alpha cells.