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Potential role of E4orf1 protein in aging-associated impairment in glycemic control

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ABSTRACT

Aging constitutes a major risk factor for the development of type-2 diabetes (T2D) where glucose tolerance declines with age, resulting in a high prevalence of T2D and impaired glucose tolerance in the elderly population. Currently more than half of the 20 million U.S. adults with T2D are above the age of 60, and the largest increase in T2D prevalence is expected in the elderly. Obesity is a causative factor for T2D associated insulin resistance and hyperglycemia. Furthermore, the aging process is accelerated by hyperglycemia and effective treatment options are limited for the vulnerable aging population. One of the mechanisms contributing to aging associated hyperglycemia is resistance to insulin-mediated glucose disposal. Chronic hyperglycemia also accelerates aging by increasing pro-inflammatory milieu leading to impaired immune function. Although currently available anti-diabetic agents improve glycemic control, they have potential serious side effects in some cases. Therefore, additional and better drugs are urgently needed for treatment of insulin resistance and aging associated health risk factors. This review presents the novel use of a microbial protein, E4orf1 as a potential anti-diabetic agent, which functions independent of insulin and obesity, highlighting the role of unique sources for future drug development.

1. Introduction

1.1. Aging

Aging is an inevitable biological process and all living organisms experience it since birth. The work by Rowe and Kahn helped progression of scientific research on aging through their concept of "successful aging". Low probability of disease and related disability, high cognitive and physical functional capacity and active engagement in life are the goals of successful aging.[1] Over the years, researchers have worked hard to come up with an optimal definition for aging but there is no precise definition yet. In recent years the term "frailty syndrome", which stands for a combination of organ dysfunction leading to increased vulnerability and adverse health outcomes with aging is seen in the literature.[2] In summary, aging can be defined as a time- dependent deterioration in physiological organ function resulting in morbidity and ultimate mortality.[3] It is important to understand that aging is an important health risk factor, which is associated with organ damage resulting in physiologically manifested conditions of disease that imposes the need for ever evolving and appropriate health care.

The specific sequence of events, leading to infirmity and death have long been a topic of research interest but many pieces of the puzzle still remain a mystery. Scientists have tried to elucidate the possible mechanisms to explain the gradual decline in organ function and death that is thought to be pre-programmed at the molecular level in living beings. One of the most broadly accepted mechanisms of aging is shortening of the ends of chromosomes, the telomeres.[4] Shortened telomeres cause genetic alterations and disturbances in the cell cycle leading to senescence.[5]

The oxidative stress theory of aging is also another contributor towards the aging process. Generation of reactive oxygen species (specifically in the mitochondria) cause DNA damage and replication stresses in an organism.[6] Exhaustion of stem cells can also contribute towards aging. Initially stem cells are protected from oxidative DNA damage over the progeny cells.[7] However, with time, stem cells also undergo DNA damage, and accumulation of unrepaired DNA alters transcription and neo-synthesis of structural and functional proteins. This impaired cell signaling causes stem cell demise and physical changes that constitute aging.[3]

It has been shown that activation of autophagy is important to extend life span in yeast, flies, nematodes and mice.[8] Kaeberlein suggests that the insulin-like signaling pathway and targets of rapamycin are potential

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paths that slow the aging process.[9] This is because when nutrients and growth stimuli are restricted, signaling along these pathways is reduced or absent and longevity is maximized. This is also consistent with the idea that reproduction and aging are evolutionarily coupled allowing living beings to withstand periods of scarcity and resume reproduction (and accelerated aging) when the circumstances are favorable.

Researchers have categorized the molecular mechanisms of aging suggesting a sequential pattern during aging. Lopez-Otin et al describe hallmarks of aging to be grouped under three categories: primary (DNA damage, telomere attrition, epigenetic alterations and loss of proteostasis), antagonistic (cellular senescence, presence of reactive oxygen species and deregulated nutrient sensing) and integrative (stem cell exhaustion and altered intercellular communication).[10] The interrelationship between these categories is explained as a gradual process over time initiated by primary factors that result in antagonistic and integrative hallmarks. Primary hallmarks impose negative effects on cellular processes, while the activity of antagonistic hallmarks depends on the intensity of their presence. Integrative hallmarks arise due to irreversible damage caused by the primary and antagonistic hallmarks that cannot be corrected by homeostasis.

The advancement in biomedical research, medicine and technology has improved medical care with a concomitant increase in the aging population living with disease. The consequence of aging is an array of human pathology including cancer, diabetes, cardiovascular disease and neurodegenerative disorders, including dementia and Alzheimer's disease (AD). The Centers for Disease Control (CDC) report that in 2015 the leading cause of death in the U.S. was heart disease. It also lists that deaths from stroke, AD, and T2D follow closely at 5th, 6th and 7th positions, respectively. It is important to note that these pathological conditions and diseases are prevalent in the aging population and are manifested over a period of time with the gradual process of aging. According to the 2014 report of the U.S. Department of Health and Human Services, the aged population (65 years and above) is estimated to be 14.5% of the population and it is projected to rise to 21.7% by 2060. With an expected rise in the aging population, the burden of offering optimal health care, intervention, and cost is also expected to increase. This poses as a challenge to cope in the future, which highlights the importance of understanding complex aspects of aging and related pathology.

1.2. Aging and glucose metabolism

It is important to note that many of the theories that explain aging agree that impairment of insulin signaling is a potent contributor to accelerated aging. Studies show that oxidative stress is a contributor to insulin resistance. Accumulation of free radicals and deterioration of antioxidant mechanisms cause cellular, and organelle damage with increased lipid peroxidation leading to enzyme degradation, thus initiating a state of glucose intolerance that over time leads to diabetes mellitus.[11] Mitochondrial overproduction of reactive oxygen species (superoxide radicals) leads to activation of five molecular pathways (polyol flux, formation of advanced glycation end products, increased expression of receptors, and activating ligands of advanced glycation end products, activation of protein kinase C isoforms, and overactive hexosamine pathway) that inactivates anti-atherogenic enzymes leading to complications of diabetes.[12] Exposure of hydrogen peroxide (free radicals resulting from mitochondrial over production) activates stress kinases (c-Jun N-terminal kinase, p38, and extracellular receptor kinases) that down regulate cellular insulin response reducing insulin stimulated glucose uptake into cells, glycogen synthesis, and protein synthesis.[13] IGF-1(insulin- like growth factor 1) and the insulin signaling pathway, collectively known as the "insulin and IGF-1 signaling" pathway (IIS) is considered a mediator of aging.[10]

Aging itself results in pathology of various biological systems leading to metabolic syndrome. This syndrome is a collection of conditions including diabetes, hyperinsulinemia, insulin resistance, hypertension, and lipid abnormalities, and is majorly related to visceral adiposity in the elderly population.[14,15] The metabolic syndrome of aging was originally called insulin resistance syndrome as insulin resistance was the trigger leading to metabolic syndrome.[16] Dysfunctional IRS-PI3-kinase/Akt pathway causes impaired glucose uptake in muscle and fat cells and also leads to failure in suppression of hepatic glucose production. This condition of relative insulin resistance is a contributor to impaired lipid uptake and storage of circulating lipids resulting in elevated levels of very low density lipoproteins, which contribute to cardiovascular events.[17] With advanced age, fat tissue distribution changes from subcutaneous to intra- abdominal visceral depots and to ectopic sites such as muscle, liver and bone marrow.[18]

1.3. Aging and obesity

The National Health and Nutrition Examination Survey (NHANES) report of 2011–2014 shows that in the United States 36% of adults and 17% of youth have obesity. The report further shows that 1999–2000 through 2013–2014, there has been a significant increase in obesity among both adults and the younger population. The increasing rates of obesity are becoming a challenge to the health care system.[19] The current youth if not given proper nutritional guidance and intervention techniques, will gradually grow into adults with a burden of obesity associated health conditions. This is further exaggerated by the fact that life expectancy is also increasing with advancement in pharmaceutical interventions and medical knowledge. According to a report published in the United States Census Bureau, there is a growth in the older population as the baby boomers started to turn 65 years old in 2011 and by 2050 they would be over 85 years old thus causing the older population to double in 2050 from the estimates in 2012.[20]

Aging and obesity are important risk factors affecting the well being of the future population. It is important to point out that with progression of life there is redistribution of body fat in individuals, which is age dependent. Relative increase in intra-abdominal fat and increases in intramuscular and intrahepatic fat in older individuals are associated with insulin resistance.[21–23] Epidemiological studies show that there is an increase in BMI until around 60 years of age after which there is no change or decrease in BMI,[23] the latter being biased by higher mortality rate among adults with obesity. According to the World Health Organization, some of the major contributors of the premature mortality with obesity are attributed to pathological consequences such as cardiovascular disease, T2D, osteoarthritis and cancer (endometrial, breast, and colon). Obesity alone is implicated in cardiac hypertrophy and congestive cardiac failure.[24] Prospective studies show that accumulation of visceral fat is an independent risk factor for development of insulin resistance and T2D.[16]

In the context of an increasing aged population, obesity mediated insulin resistance and glucose intolerance that develops with age, augments morbidity and mortality. This highlights the importance and need for novel treatment options to address this trend that is visible as a future burden to health care. Aging is irreversible and the only component that humans may be able to slow down would be excess weight gain and obesity. Current treatment options for obesity emphasize weight loss techniques, which include self-motivated transformation of ones self. Some randomized controlled trials suggest that modest weight loss is obtained with exercise alone while others show high level of physical activity (when volume of exercise prescribed is equivalent to energy deficit accounting for weight loss by caloric restriction and when there is constant energy intake) leads to significant weight loss.[25] Randomized controlled trials also show that even with one on one interactive weight control monitoring over a period of 24 months, only 41.6% individuals are able to obtain a 5% or more loss in body weight.[26] The lack of adequate weight loss or its maintenance may lead back to the vicious cycle of unwanted consequences such as the metabolic syndrome. Other treatment options available for obesity treatment in the form of pharmacology and surgery also have the same challenge of weight management or keeping off the lost weight.[27]

2. Aging, obesity and glucose intolerance

Fig. 1 shows the relationship between aging, obesity and glucose intolerance, which is the focus of this review. Aging is inevitable, and as a consequence, there is metabolic derangement in the body leading to deterioration in glucose metabolism. Additionally, with glucose intolerance there is a progression of the aging process, although the specific reasons are not fully understood and discussed above.

Limited mobility with age and redistribution of body fat may cause visceral obesity, which is also implicated in increased morbidity and mortality as indicated by the National Health and Nutrition Examination Survey I (NHANES I) and its Epidemiologic Follow-Up Study (NHEFS).[28] Obesity is a risk factor for developing glucose intolerance, which leads to insulin resistance. All these factors ultimately form an interconnected vicious circle that exerts great public health care concern not only in the United States, but all over the world.

2.1. Novel treatment options are needed in the future

Considering the relationship between aging, obesity, and glucose intolerance and the challenges in achieving effective weight loss for the majority, a novel treatment option in the future should be one, which can improve impaired glucose intolerance in spite of obesity and age. In particular, the proximal insulin-signaling pathway, identified by insulin receptor (IR) and insulin receptor substrate1/2 (IRS1/2), which is often impaired in individuals with glucose intolerance.[27,29,30] Most of the currently available medications such as metformin, sulphonylureas, and thiazolidinediones, are either insulin secretagogues, mimetics, or sensitizers.[31]and are dependent on the action of this proximal insulin-signaling pathway. An agent that may be able to bypass the proximal insulin-signaling pathway would be beneficial in improving glucose intolerance.

3. Adenovirus 36 (Ad36) and its E4orf1 protein as potential anti-diabetic therapy

A human adenovirus belonging to subgroup D and serotype 36 (Ad36), was first isolated in 1978 from the fecal sample of a 6 year-old diabetic girl in Germany, suffering from enteritis.[32] Experimental Ad36 infection significantly increases adiposity in chicken, mice, rats or marmosets compared with respective uninfected controls,[33–36] leading to a paradoxical lowering of serum triglycerides and cholesterol. In rats, Ad36 improved HOMA-IR and despite increased adiposity, maintained euglycemia with approximately half the fasting serum insulin levels of control rats.[36] In mice and rats, Ad36 infection improved glycemic control, as measured by glucose tolerance test (GTT), fasting glucose and serum insulin levels when exposed to either chow or 60%

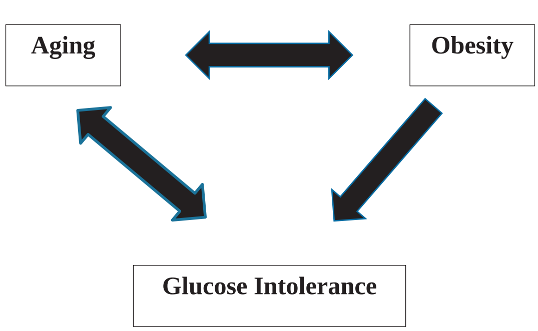

Fig. 1. Relationship between aging, obesity and glucose intolerance.

high fat diet.[36,37] Additionally, Ad36 significantly attenuated hepatic lipid accumulation in these mice.[37]

The molecular mechanisms underlying improvement in glycemic control by Ad36 was examined in studies with adipose tissue explants or primary preadipocytes (human adipose tissue derived stem cells; hASC)[38] and human primary skeletal muscle cells (hSKMC) obtained from non-diabetic or diabetic subjects, which showed Ad36 dose dependently increases glucose uptake in cells.[39] In hASC and hSKMC, Ad36 decreases the phosphorylation of IR, IRS1 tyrosine phosphorylation, and conversely increases serine phosphorylation, and reduces IRS1 and IRS2 associated PI3K activities,[37–39] which collectively indicate an inhibition of the proximal insulin signaling. Despite this, Ad36 increases glucose uptake in these cells and adipose tissue explants[38,39] by robustly upregulating the distal insulin signaling, independent of the proximal signaling. In fact, siRNA mediated knockdown of IR did not affect Ad36-induced increase in glucose uptake by 3T3-L1 cells[40] as Ad36 upregulated glucose transporter 4 (Glut4) and glucose uptake *via* a Ras-dependent activation of distal insulin signaling (the PI3K-AKT pathway).[38]

However, to harness this beneficial effect of Ad36 for humans would require infecting people with the virus, which is not ethical. Subsequent *in vitro* studies identified E4orf1, a 125 amino acid protein of Ad36 as necessary and essential for the observed beneficial effects with Ad36. *In vitro* and *in vivo* studies collectively show E4orf1 increases glucose uptake in cells[41] and improves glucose clearance in mice.[42,43] In cells, under conditions of impaired insulin receptor and IRS1, and the proximal insulin signaling, E4orf1 up-regulates the distal insulin signaling pathway *via* increased expression of PI3K, PKB/AKT, and GLUT4 translocation to increase cellular glucose uptake.[44] Improvement in glucose uptake was also observed in cells even in the presence of inflammatory cytokines.[45] In hepatocytes, E4orf1 reduces glucose output under basal and gluconeogenic conditions, reduces de-novo lipogenesis, increases complete fatty acid oxidation and promotes lipid transport,[46] which collectively are the key determinants of hepatic lipid storage. Together, the *in vitro* studies show the ability of E4orf1 to improve cellular glucose metabolism when insulin resistance is mimicked with impaired insulin signaling and presence of inflammation. Similarly, in mice, retrovirus mediated expression of E4orf1 reproducibly improved glucose excursion following a glucose load despite a high fat diet.[42] E4orf1 also modulated molecular signaling in mice tissue, which included greater protein abundance of adiponectin, p-AKT, and glucose transporter Glut4. Transgenic expression of E4orf1 in the adipose tissue of mice improved glucose clearance despite a high-fat diet challenge by enhancing the Ras-ERK-MAPK signaling in transgenic adipocytes as a potential alternative route to bypass proximal insulin signaling.[43] Hepatic expression of AAV-E4orf1 in a genetic model of diabetes (db/db mice), dietary model of insulin resistance (DIO mice), and normoglycemia (wild-type mice) completely alleviated hyperglycemia and robustly improved glycemic control without significantly increasing hepatic steatosis.[47] Recently Yoon, et al, showed, hydrodynamic gene delivery of E4orf1 with an adipocyte-targeting sequence (ATS-E4orf1) in the liver of high-fat fed and streptozotocin-injected mice (disease models of type 2 and type 1 diabetes, respectively), improved the ability of these mice to eliminate excess glucose from the blood and ameliorate liver function in both disease models.[48] Therefore, E4orf1 expression, systemic or tissue specific, in mice improves glycemic control independent of high fat diet intake and impaired insulin signaling, which are often associated with obesity, insulin resistance, and diabetes (Fig. 2).

3.1. Implications for E4orf1 as a therapeutic agent in improving aging-associated pathogenesis of diabetes

There are several factors that contribute to the high prevalence of diabetes in the aging population, such as alterations in glucose-induced insulin release and resistance to insulin-mediated glucose disposal, that interact with genetic background to explain the progressive

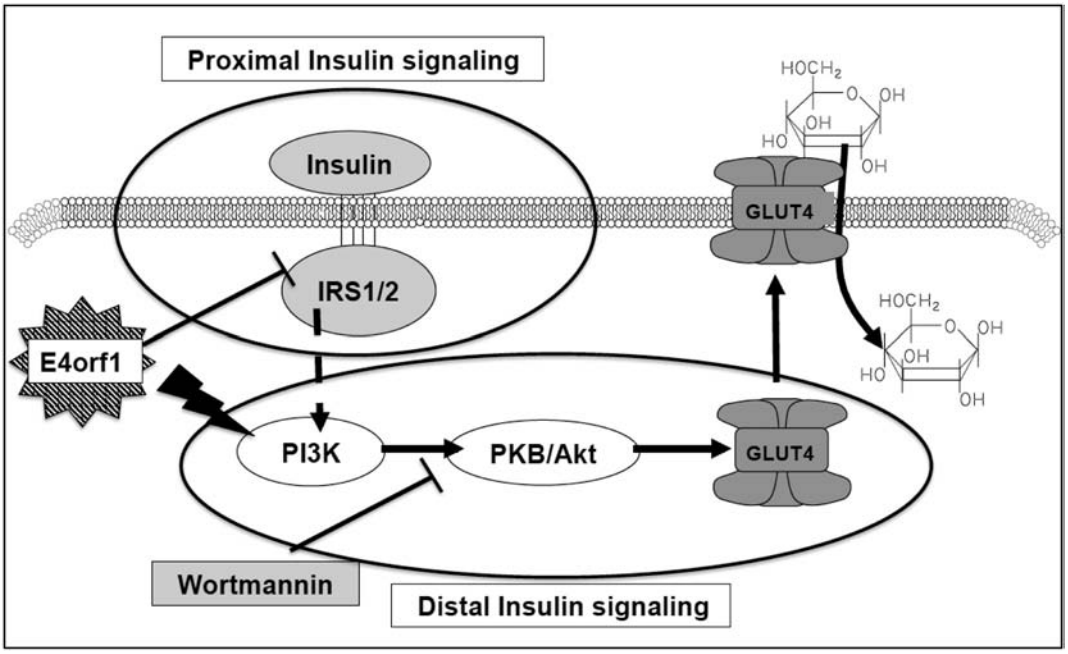

Fig. 2. E4orf1 increases cellular glucose uptake by activating the distal insulin signaling: E4orf1 expression blocks the proximal insulin signaling by increasing serine phosphorylation of insulin receptor and insulin receptor substrate 1/2 (IRS1/2), while increasing cellular glucose uptake by activating Ras/PI3K mediated phosphorylation of PKB/Akt leading to translocation of Glut4 from the cytoplasm to the cell membrane, collectively known as the distal insulin signaling.

increase in the incidence of diabetes with aging. Lifestyle factors are also important. Individuals who are obese (especially if the distribution of body fat is central), who consume diets that are high in saturated fat and low in complex carbohydrates, or who are inactive are more likely to develop diabetes as they age.

A number of studies have carefully evaluated glucose metabolism in middle-aged patients with T2D and shown that patients have several metabolic abnormalities, including increased fasting hepatic glucose production, altered glucose-induced insulin release, and marked resistance to insulin-mediated glucose disposal, which are markedly significant with the comorbid presence of obesity.[49–52] As a potential therapeutic agent for diabetes, E4orf1 has an insulin sparing effect,[27] and insulin independent action for glucose disposal,[40,43] suggesting E4orf1 could possibly overcome the observed altered glucose-induced insulin release, and resistance to insulin-mediated glucose disposal. Furthermore, E4orf1 is able to improve glycemic control despite obesity,[42,43,47] and prevents hepatic steatosis and glucose output from the liver.[43,46] Increased production of tumor necrosis factor α (TNF-α), a cytokine produced by adipocytes during obesity is believed to contribute to the pathogenesis of insulin resistance.[53] In contrast, *in vitro* expression of E4orf1 in the presence of inflammatory cytokines, TNF-α and monocyte chemoattractant protein (MCP-1) is able to increase cellular glucose uptake.[45] T2D is also characterized by defects in β-cell function that may become more manifest later in life. The safety of therapies in older adults with diabetes is important to consider in clinical practice to minimize polypharmacy and potential adverse side effects. *In vivo*, E4orf1 expressing mice do not display the transient insulin spike typically observed during glucose tolerance tests (GTT),[43] indicating reduced insulin secretion in response to exogenous glucose and may protect pancreatic beta cells from exhaustion and damage. Finally, the risks of overtreatment of hyperglycemia in older adults are significant and include hypoglycemia and increased treatment burden. Age may affect counter regulatory responses to hypoglycemia.[54] Alternatively, insulin supplementation treatment of autoimmune phenomena like type1 diabetes could also contribute to fatal hypoglycemia. E4orf1 has a distinct advantage in treating hyperglycemia associated with T2D, as it does not cause severe fasting hypoglycemia,[42,43,55] and in the presence of excess exogenous glucose neither does E4orf1 display uncontrolled glucose clearance. Furthermore, the insulin sparing ability of E4orf1 makes its use suitable for even type1 diabetes.[48]

3.2. Future of Ad36E4orf1 research

Previous proof of concept studies using transgenic and AAV/retrovirus expression[42,43,47,48] highlights the potential of Ad36E4orf1 protein as a novel anti-diabetic agent. However to use E4orf1 as a therapeutic option in humans, better and effective delivery routes need to be developed. Some possible choices could be increasing bioavailability of E4orf1 in metabolic tissues *via* nano-particle mediated peptide delivery or developing chemical analog drug delivery. Collectively, Ad36E4orf1 appears to be a promising and effective therapeutic agent to improve age associated glucose intolerance without the requirement of significant weight loss.

4. Conclusion

Obesity and impaired glycemic control are associated with the aging process forming a vicious cycle. As aging is inevitable, comprehensive lifestyle modification based on reduced energy intake and/or increased physical activity, or bariatric surgery could improve associated glycemic impairment and the ill effects of aging. However, effective and long-term obesity treatment is highly challenging and also the current anti-diabetic drugs require functional proximal insulin signaling for their action. Hence, Ad36E4orf1 could be a novel approach to effectively improve glycemic control despite obesity and impaired proximal insulin signaling.

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Nikhil V. Dhurandhar has several patents in viral obesity and adenovirus 36 including uses for E1A, E4-ORF1 gene and protein, and AKT1inhibitor, and has received grant support for determining anti-diabetic properties of E4-ORF1 protein.

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