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The Lipid Peroxidation End-Product 4-Hydroxynonenal induces insulin resistance in isolated fat slow-twitch skeletal muscle

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Abstract:

A primary defect leading to the development of type 2 diabetes is insulin resistance of the glucose transport system in skeletal muscle. One factor known to induce insulin resistance is oxidative stress. A by-product of lipid peroxidation is the reactive aldehyde 4-hydroxynonenal (4-HNE), an oxidant that induces a number of deleterious consequences on cell function. However, the impact of 4-HNE on the glucose transport system in rat slow-twitch skeletal muscle is currently not known. Therefore, we assessed the impact of 4-HNE on insulin signaling factors (IRS-1 protein expression and phosphorylation of Akt Ser473 (pAkt) and AS160 Thr642 (pAS160)) and on glucose transport activity in mammalian slow-twitch muscle. Strips of soleus muscle from lean Zucker rats were incubated with 4-HNE (50 μM) in the absence or presence of insulin (5 mU/ml) for up to 6 hr. Insulin-stimulated glucose transport activity (determined using 2-deoxyglucose uptake) was decreased by 4-HNE at 2 hr (30%), 4 hr (26%), and 6 hr (39%) (all $p<0.05$). At 2 hr of 4-HNE treatment in the presence of insulin, pAS160 was decreased by 28%, whereas pAkt was only reduced 11% and IRS-1 protein levels were not changed. At 4 hr, pAS160 was decreased by 22%, as was pAkt, and IRS-1 levels were 39% lower than in the control muscles. At 6 hr, pAS160 was 47% lower, pAkt was decreased by 26%, and IRS-1 protein levels were reduced by 51%. Interestingly, IRS-2 protein levels were decreased by 17% only at the 6 hr time point. In summary, these data indicate that the lipid peroxidation end-product and oxidant 4-HNE induces insulin resistance of glucose transport activity in rat slow-twitch skeletal muscle, initially associated with impaired phosphorylation (and therefore reduced activation) of AS160. Longer durations of 4-HNE exposure led to a greater impairment of Akt phosphorylation and to a selective loss of IRS-1 protein. These results provide further support for an important role of oxidative stress in the etiology of skeletal muscle insulin resistance.

THE LIPID PEROXIDATION END-PRODUCT 4-HYDROXYNONENAL INDUCES INSULIN RESISTANCE IN ISOLATED RAT SLOW-TWITCH SKELETAL MUSCLE

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Abstract

A primary defect leading to the development of type 2 diabetes is insulin resistance of the glucose transport system in skeletal muscle. One factor known to induce insulin resistance is oxidative stress. A by-product of lipid peroxidation, which is elevated in type 2 diabetes, is the reactive aldehyde 4-hydroxynonenal (4-HNE), an oxidant that induces a number of deleterious consequences on cell function. However, the impact of 4-HNE on the regulation of the glucose transport system in mammalian slow-twitch skeletal muscle is currently not known. Therefore, we assessed the impact of 4-HNE on insulin signaling factors (IRS protein expression and phosphorylation of Akt Ser^{473} (pAkt) and AS160 Thr^{642} (pAS160)) and on glucose transport activity in rat slow-twitch muscle. Strips of soleus muscle from lean Zucker rats were incubated with 4-HNE (50 μM) in the absence or presence of insulin (5 mU/ml) for up to 6 hr. Insulin-stimulated glucose transport activity (determined using 2-deoxyglucose uptake) was decreased by 4-HNE at 2 hr (30%), 4 hr (26%), and 6 hr (39%) (all $p<0.05$). At 2 hr of 4-HNE treatment in the presence of insulin, pAS160 was decreased by 28%, whereas pAkt was minimally reduced (11%) and IRS-1 and IRS-2 protein levels were not changed. At 4 hr, pAS160 was decreased by 22%, as was pAkt, and IRS-1 levels were 39% lower than in the control muscles. At 6 hr, pAS160 was 47% lower, pAkt was decreased by 26%, and IRS-1 protein levels were reduced by 51%. Interestingly, IRS-2 protein levels were decreased by 17% only at the 6 hr time point. In summary, these data indicate that the lipid peroxidation end-product and oxidant 4-HNE induces insulin resistance of glucose transport activity in rat slow-twitch skeletal muscle, initially associated with impaired phosphorylation (and therefore reduced activation) of AS160. Longer durations of 4-HNE exposure lead to a greater impairment of Akt phosphorylation and to a selective loss of IRS-1 protein. These results provide further support for an important role of factors associated with elevated oxidative stress in the etiology of skeletal muscle insulin resistance.

Introduction

The human body produces many hormones to maintain glucose homeostasis; a critical hormone in this regard is insulin, which is produced in and secreted from the beta cells of the pancreas. Insulin can act on skeletal muscle, fat tissue, liver, and specific neurons in the brain to facilitate glucose homeostasis[1,6]. In skeletal muscle, insulin causes glucose to be removed from the blood and stored as glycogen. This glucose transport into skeletal muscle cells is regulated by the canonical insulin signaling cascade (e.g. IRS/Akt/AS160 axis)[7]. The insulin signaling pathway is activated when insulin binds to the insulin receptor. Following this activation of the insulin receptor tyrosine kinase, the insulin receptor substrate (IRS) is phosphorylated[16]. Several downstream factors are subsequently engaged, a major one being the serine/threonine kinase called Akt (also known as Protein Kinase B)[16]. An important substrate of Akt is AS160, an inhibitor of the GTP-binding protein Rab that is needed for GLUT4 translocation[2,10]. Phosphorylation of AS160 on Thr^{642} by Akt inhibits the action of AS160, allowing for GLUT4 translocation[2]. When this insulin signaling pathway is disrupted, insulin resistance can occur, inhibiting glucose transport activity and leading to a vast number of detrimental effects, including hypertension, polycystic ovarian disease, obesity, atherosclerosis, and most importantly, an increased risk of the development of type 2 diabetes[15].

Type 2 diabetes, a chronic disease affecting over 25 million people[12], is characterized by elevated levels of glucose in the blood (for example, a fasting blood glucose concentration above 126 mg/dl). This metabolic disorder develops as a result of two specific defects: one pathophysiological development is that the insulin-sensitive cells in the body do not respond normally to the insulin being produced (insulin resistance), and the other is that the beta cells of the pancreas fail to produce and secrete enough insulin to overcome the insulin resistance[1]. This illness is more likely to develop in those who have high blood pressure and cholesterol, those who have impaired fasting glucose, and those who are overweight[12]. Individuals suffering from this illness are plagued with many complications, such as cardiovascular disease, nerve, foot, eye, and kidney damage, and the increased risk of osteoporosis and Alzheimer's disease[1].

There are several contributing factors to the development of insulin resistance, one of which is oxidative stress (overproduction of reactive oxygen species)[7]. When this is coupled with reactive nitrogen species, nitrosative stress can also occur[9]. Nitrosative stress-associated insulin resistance is often accompanied by dyslipidemia (an abnormal amount of various lipids in the bloodstream)[9].

The overabundance of reactive species in the body is associated with increased lipid peroxidation, a process whereby free radicals remove electrons from lipids in the cell membrane, resulting in cellular damage[3]. A consequence of this process is the enhanced production of the lipid peroxidation end-product and reactive aldehyde 4-hydroxynonenal (4-HNE)[3]. Specifically, cellular plasma membrane lipids, such as phospholipids, become oxidized by reactive oxygen species. When this occurs, one of the by-products is 4-HNE (which is itself an oxidant)[11] (Fig. 1). It is known that insulin action in muscle cells can become impaired due to oxidative damage of proteins, as well as from oxidative stress, when impacted by 4-HNE[13]. To date, it has been

demonstrated that 4-HNE can induce insulin resistance in adipocytes[3] and in mouse fast-twitch gastrocnemius skeletal muscle[13]. However, the impact of 4-HNE on the glucose transport system in rat slow-twitch skeletal muscle is currently unknown.

Therefore, in the present study we assessed the impact of 4-HNE on critical insulin signaling factors (IRS protein expression and phosphorylation of Akt Ser^{473} (pAkt) and AS160 Thr^{642} (pAS160)) and on glucose transport activity in slow-twitch skeletal muscle (soleus) from the lean Zucker rat to determine if this lipid peroxidation end-product and oxidant can impair these insulin signaling factors and induce insulin resistance of glucose transport activity.

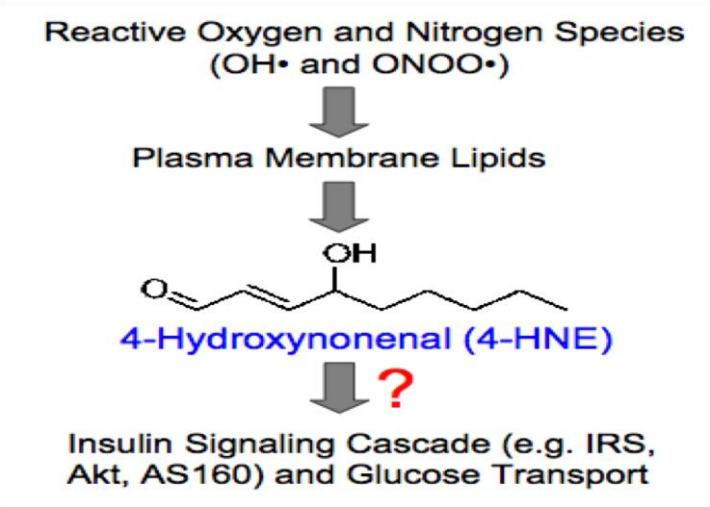

Figure 1: Production of 4-HNE and potential impact of 4-HNE on insulin signaling and glucose transport activity in slow-twitch muscle.

Methods

Animals. All procedures used were approved by the Institutional Animal Care and Use Committee at the University of Arizona. Female lean Zucker rats (Harlan, Indianapolis, IN) were used at 6–8 weeks of age (body weights of 120-140 g). Animals were housed in a temperature-controlled (20–22 °C) room with a 12:12-h light–dark cycle, and the animals had free access to chow (Teklad 7001, Madison, WI) and water. At 5 PM the evening before each experiment, animals were restricted to 4 g of chow, which was consumed immediately. Experiments began between 8 and 9 AM the next morning.

Muscle incubations. Animals were deeply anesthetized with pentobarbital sodium (50 mg/kg), and strips of soleus muscle (<25–35 mg) were prepared for in vitro incubation in the unmounted state. Muscles were initially incubated for 2–6 h at 37 °C in oxygenated (95% O_2/5% CO_2) Krebs–Henseleit buffer (KHB) containing 8 mM glucose, 32 mM mannitol, and 0.1% bovine serum albumin (Sigma Chemical, St. Louis, MO), in the absence or presence of 50 µM 4-HNE (Sigma Chemical, St. Louis, MO) with or without 5 mU/ml insulin (Humulin, Eli Lilly,

Indianapolis, IN). After the initial incubation period, the muscles were rinsed for 10 min at 37 °C in 3 ml of oxygenated KHB containing 40 mM mannitol, 0.1% BSA, and 4-HNE and/or insulin, if present previously. Following the rinse period, muscles were transferred to 2 ml of KHB containing 1 mM 2-deoxy-[1,2-^{3}H]glucose (0.3 mCi/mmol; Sigma Chemical), 39 mM [U-^{14}C] mannitol (0.8 mCi/mmol; ICN Radiochemicals, Irvine, CA), 0.1% BSA, and 4-HNE and/or insulin, if previously present, and incubated for 20 min at 37 °C. At the end of this final incubation period, muscles were removed and quickly frozen in liquid nitrogen, weighed, and placed in 0.5 ml of 0.5 mM NaOH. After the muscles were completely solubilized, 5 ml of scintillation cocktail were added, and the specific intracellular accumulation of 2-[^{3}H]DG was determined as described previously[5].

Insulin signaling. Soleus muscle strips were incubated for up to 6 hr without or with 4-HNE (50 μM)[3,13] in the absence or presence of insulin (5 mU/ml). Homogenates of soleus muscle were used for analyzing insulin signaling by immunoblotting with commercially available antibodies. Muscles were frozen after the initial incubation period, weighed, and stored at −80°C until analysis. Muscles were homogenized in eight volumes of ice-cold lysis buffer (50 mM HEPES, 150 mM NaCl, 20 mM Na pyrophosphate, 20 mM β-glycerophosphate, 10 mM NaF, 2 mM Na_3VO_4, 2 mM EDTA, 1%Triton X-100, 10% glycerol, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 0.5 μg/ml pepstatin, and 2 mM PMSF). Homogenates were incubated on ice for 20 min and then centrifuged at 13,000*g* for 20 min at 4°C. Total protein concentration was determined using the BCA method (Pierce, Rockford, IL). Samples containing equal amounts of total protein were separated by SDS–PAGE on 10% or 12% polyacrylamide gels and transferred to nitrocellulose. Membranes were incubated overnight with antibodies against phosphorylated Akt Ser^{473} or AS160 Thr^{642} (Cell Signaling Technology, Danvers, MA), or overnight with antibodies against IRS-1, IRS-2, total Akt, or total AS160 (Cell Signaling).

The membranes were then incubated with secondary goat anti-rabbit antibody conjugated with horseradish peroxidase (HRP) (Chemicon, Temecula, CA) or anti-mouse antibody conjugated with HRP (Santa Cruz Biotechnology, Santa Cruz, CA). Proteins were visualized using a Bio-Rad Chemidoc XRS instrument (Bio-Rad Laboratories, Hercules, CA) using the SuperSignal West Femto Maximum Sensitivity Western blot detection substrate (Pierce). Band density was quantified using the Bio-Rad Quantity One software.

Statistical analysis. All values are expressed as means ± SE for 4 muscles/group. Differences between mean values in the absence or presence of 4-HNE (performed using paired strips of muscle from the same whole muscle) were determined by the paired Student's t-test. A value of $p<0.05$ is considered to be statistically significant.

Results

Effects of 4-HNE on glucose transport activity. The soleus muscles were incubated in 50 μM 4-HNE in the absence or presence of insulin for 2, 4, or 6 hours. There was no change due to 4-HNE in the absence of insulin. In the presence of insulin, 4-HNE significantly decreased

($p<0.05$) basal glucose transport levels at 2, 4, and 6 hr. Decreases in insulin-stimulated glucose transport occurred at 2 hr (30%), 4 hr (26%) and 6 hr (39%) (all $p<0.05$) (Fig. 2).

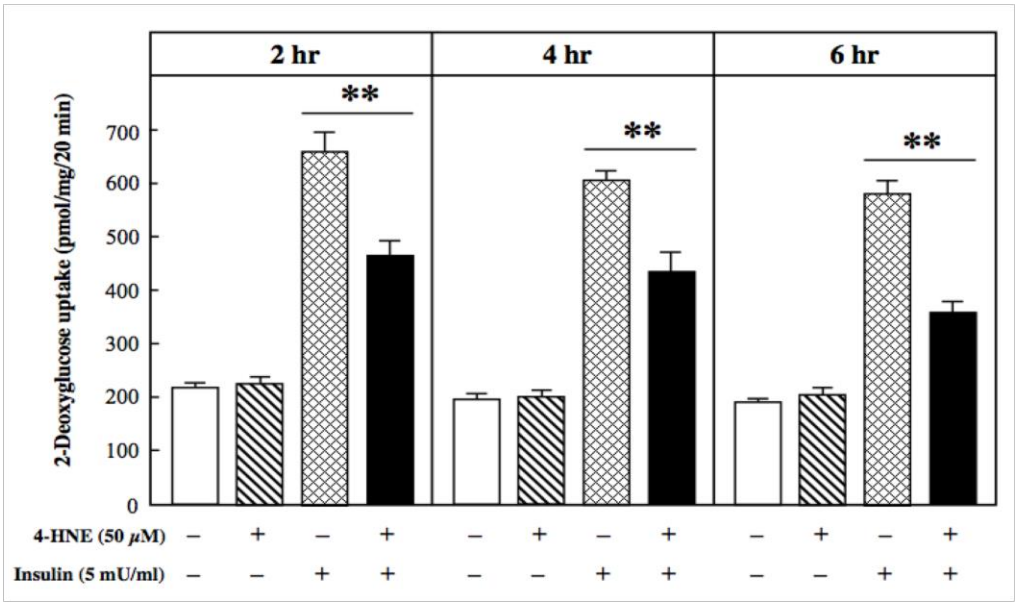

Figure 2: Effects of 4-HNE on glucose transport activity in the absence or presence of insulin at 2, 4, and 6 hours in lean rat soleus muscle. **p<0.05 vs. insulin without 4-HNE.

Effect of 4-HNE on basal and insulin-stimulated Akt Ser^{473} phosphorylation. There was no significant change due to 4-HNE in the absence of insulin. 4-HNE treatment in the presence of insulin caused a slight decrease in pAkt at 2 hr (11%), with more robust decreases at 4 hr (22%), and 6 hr (26%) (Fig. 3).

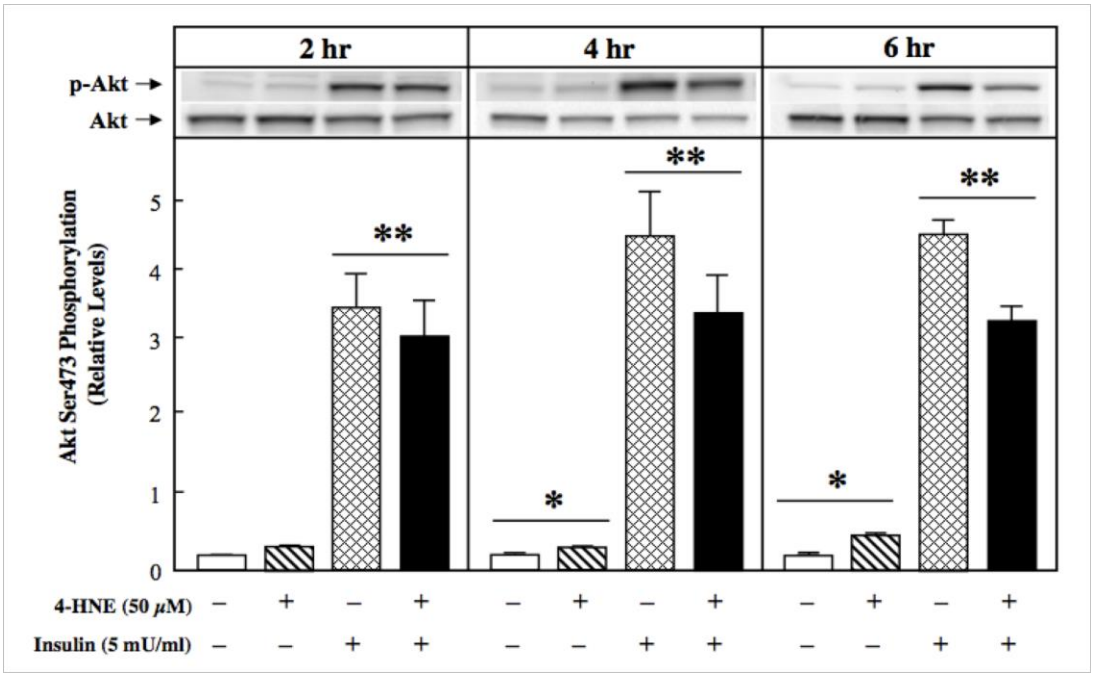

Figure 3: Effect of 4-HNE on basal and insulin-stimulated Akt Ser^{473} phosphorylation at 2, 4, and 6 hours in lean rat soleus muscle. **p<0.05 vs. no 4-HNE.

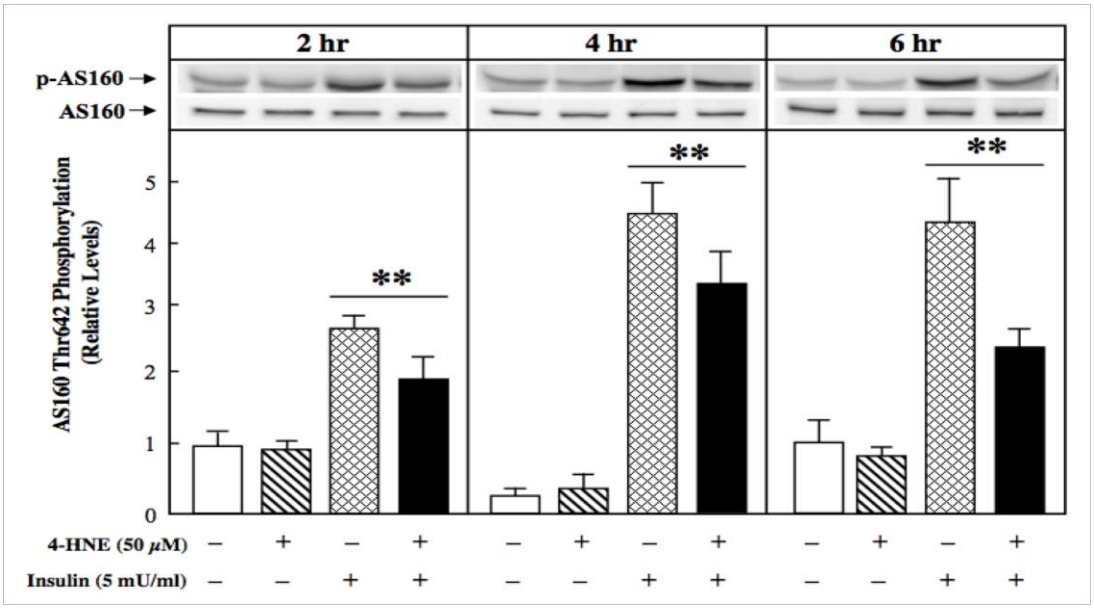

Figure 4: Effect of 4-HNE on AS160 phosphorylation (Thr^{642}) in the absence or presence of insulin at 2, 4, and 6 hours in lean rat soleus muscle. **p<0.05 vs. insulin without 4-HNE.

Effects of 4-HNE on AS160 Thr^{642} phosphorylation. As with pAkt, 4-HNE treatment did not affect pAS160 in the absence of insulin (Fig. 4). In contrast, 4-HNE treatment in the

presence of insulin led to substantially diminished pAS160 at 2 hr (28%), 4 hr (22%), and 6 hr (47 %).

Effects of 4-HNE on protein expression of IRS-1 and IRS-2. 4-HNE treatment in the presence of insulin did not alter IRS-1 protein expression at 2 hr, but IRS-1 protein levels were decreased at 4 hr (39%) and 6 hr (51%) (Fig. 5). IRS-2 protein expression in the presence of insulin was decreased slightly (17%) only at the 6 hr time point (Fig. 6).

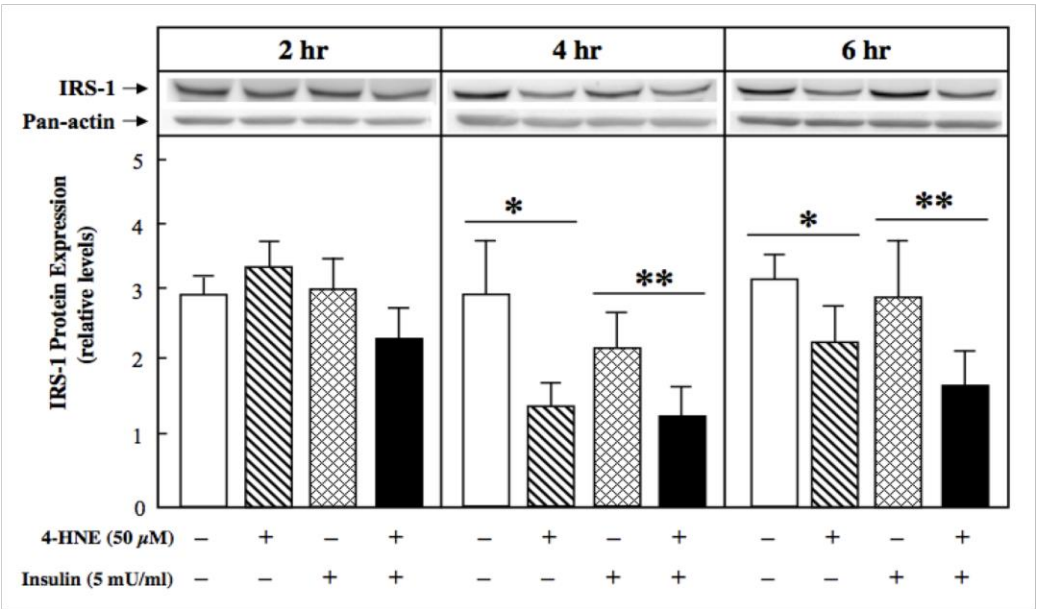

Figure 5: Effects of 4-HNE on protein expression of IRS-1 in the absence or presence of insulin at 2, 4, and 6 hours in lean rat soleus muscle. **p<0.05 vs. no 4-HNE; **p<0.05 vs. insulin without 4-HNE.

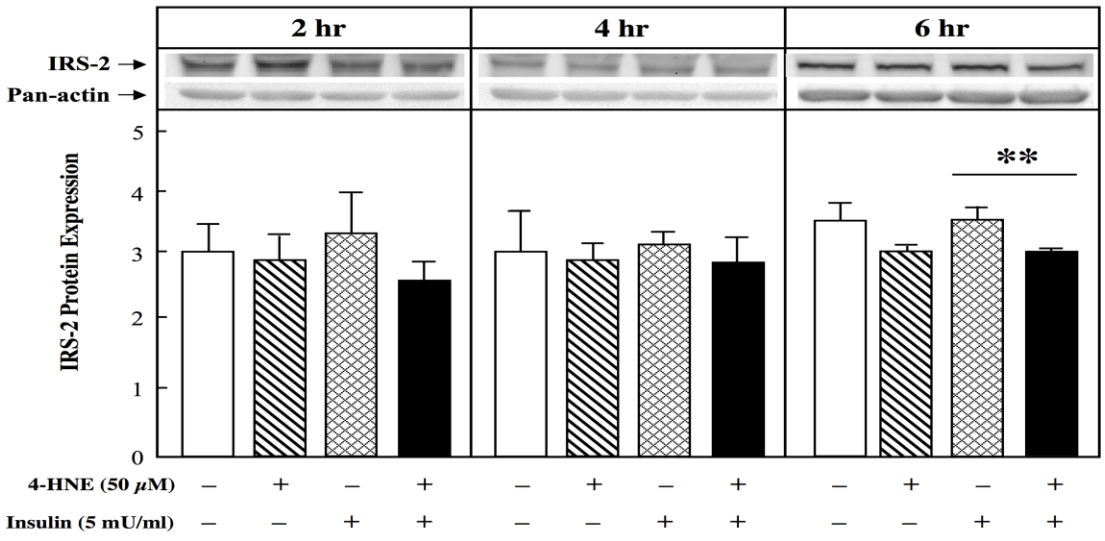

Figure 6.

Figure 6: Effects of 4-HNE on protein expression of IRS-2 in the absence or presence of insulin at 2, 4, and 6 hours in lean rat soleus muscle. **p<0.05 vs. insulin without 4-HNE.

Discussion

This research study clearly demonstrated the impact of the lipid peroxidation end-product 4-HNE on the glucose transport system in the slow-twitch soleus skeletal muscle of rats. The results of the present study revealed that in vitro 4-HNE treatment in the presence of insulin caused significant decreases in glucose transport activity, pAkt, and pAS160 after 2, 4, and 6 hours of exposure in isolated slow-twitch soleus muscle of lean Zucker rats. IRS-1 protein was decreased at 4 and 6 hours and IRS-2 was decreased slightly only at six hours. The most prominent change was in pAS160, with a 28% decrease at 2 hours, a 22% decrease at 4 hours, and a 47% decrease at 6 hours, leading to the concept that this is a key site of regulation. Insulin resistance appears to be less apparent in upstream effectors, such as pAkt and IRS-1 protein expression, as these are markedly less affected at the earliest time point (2 hours), when insulin resistance of glucose transport activity due to 4-HNE is already well established. However, the loss of IRS protein appears important for the maintenance and exacerbation of the 4-HNE-associated insulin resistance, as 4-HNE treatment in the presence of insulin resulted in a 39% decrease in IRS-1 protein expression at 4 hours and a 51% decrease at six hours, even though it was unaffected at 2 hours.

Previous investigations of the effect of 4-HNE on insulin action have been conducted in different experimental models of insulin-sensitive cells and tissues. It has been shown that 4-

HNE induces insulin resistance in cultured 3T3-L1 fat cells[3] and more recently in mouse fast-twitch gastrocnemius skeletal muscle[13].

Skeletal muscle is made up of three main fiber types: fast glycolytic (FG), fast glycolytic oxidative (FOG), and slow oxidative (SO). Insulin sensitivity in muscle appears to be a function of its fiber type composition[8]. The soleus was important to use in the present investigation because this muscle consists predominately of SO fibers[14]. As stated above, a previous study utilized the fast-twitch gastrocnemius skeletal muscle, but not slow-twitch muscle, from mice[13]. SO fibers are the most insulin-sensitive fiber type, which is why the fiber type specificity of the response to 4-HNE effects on insulin signaling and glucose transport activity needed to be addressed. With the present investigation, we extended the body of knowledge in the scientific literature by investigating the impact of 4-HNE on slow twitch skeletal muscle, adding to the previous results from mouse muscle consisting of a mix of FG and FOG fibers[13].

Research from 2012 on the fast-twitch gastrocnemius in mice found that pAkt was predominately affected by 4-HNE[13]. This contrasts with one of our main findings research, that it was pAS160, and not pAkt, that was most closely associated with the initial onset of insulin resistance due to 4-HNE. AS160 is a terminal signaling element involved in GLUT-4 translocation[2,4]. Another difference was that the mouse study[13] did not report any differences in IRS protein levels due to the 4-HNE treatments, but our research indicated a maximal 51% decrease in IRS-1 protein expression. We have concluded that in rat slow-twitch skeletal muscle, upstream insulin signaling effectors likely play a subordinate role in the response to 4-HNE, which is in contrast to the results of Pillon[13]. However, it should be emphasized that both studies agree that 4-HNE plays a major role in the disruption of the insulin signaling pathway.

There were a couple limitations to the Pillon study[13], revealing why our study was needed. First, incubated gastrocnemius muscle contains fast-twitch fibers, as discussed above. More importantly, the intact mouse gastrocnemius muscle is also a fairly thick muscle, making it difficult to incubate and not have diffusion limitations. If muscles are not properly incubated, the results can be compromised and invalid[8]. Therefore, this brings into question the validity of the Pillon study.

In conclusion, one of the main defects that leads to the development of type 2 diabetes is insulin resistance in skeletal muscle. A primary finding of the present study is that the lipid peroxidation end-product and reactive aldehyde 4-HNE directly induces insulin resistance of glucose transport activity in rat slow-twitch skeletal muscle, initially associated with the reduced activation of AS160. A progressively greater impairment of Akt phosphorylation and loss of IRS-1 protein were induced by longer exposure to 4-HNE. These results reveal the negative effect that lipid-derived oxidative stress has on skeletal muscle. The findings of this study make an important contribution to the current body of knowledge of the etiology of insulin resistance in skeletal muscle, and can hopefully be used in designing interventions that will cure the detrimental effects of diabetes.

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By

FRANCHESCA JO GIOVANNINI

A Thesis Submitted to The Honors College

In Partial Fulfillment of the Bachelors degree

With Honors in

Physiology

THE UNIVERSITY OF ARIZONA

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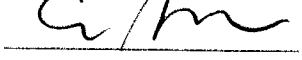

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