THUJONE, A COMPONENT OF MEDICINAL HERBS, RESCUES PALMITATE-INDUCED INSULIN RESISTANCE IN SKELETAL MUSCLE

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Running head: Thujone-mediated rescue of muscle insulin resistance

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Abstract
Thujone is thought to be the main constituent of medicinal herbs that have antidiabetic properties. Therefore, we examined whether thujone ameliorated palmitate-induced insulin resistance in skeletal muscle. Soleus muscles were incubated for \( \leq 12 \)h without or with palmitate (2mM). Thujone (0.01mg/ml), in the presence of palmitate, was provided in the last 6h of incubation. Palmitate oxidation, AMPK/ACC phosphorylation and insulin-stimulated glucose transport, plasmalemmal GLUT4, and AS160 phosphorylation were examined at 0, 6 and 12h. Palmitate treatment for 12h reduced fatty acid oxidation (-47%), and insulin-stimulated glucose transport (-71%), GLUT4 translocation (-40%), and AS160 phosphorylation (-26%), but it increased AMPK (+51%) and ACC phosphorylations (+44%). Thujone (6-12h) fully rescued palmitate oxidation and insulin-stimulated glucose transport, but only partially restored GLUT4 translocation and AS160 phosphorylation, raising the possibility that an increased GLUT4 intrinsic activity may also have contributed to the restoration of glucose transport. Thujone also further increased AMPK phosphorylation but had no further effect on ACC phosphorylation. Inhibition of AMPK phosphorylation with Adenine 9-β-D-arabinofuranoside (Ara) (2.5mM) or compound C (50µM), inhibited the thujone-induced improvement in insulin–stimulated glucose transport, GLUT4 translocation and AS160 phosphorylation. In contrast, the thujone-induced improvement in palmitate oxidation was only slightly inhibited (\( \leq 20\% \)) by Ara or compound C. Thus, while thujone, a medicinal herb component, rescues palmitate-induced insulin resistance in muscle, the improvement in fatty acid oxidation cannot account for this thujone-mediated effect. Instead, the rescue of palmitate-induced insulin resistance appears to occur via an AMPK-dependent mechanism involving partial restoration of insulin-stimulated GLUT4 translocation.

Key words: Palmitate oxidation, Glucose transport, AMPK, ACC.
Insulin resistance is a cardinal feature of type 2 diabetes, and skeletal muscle is a key tissue that contributes to regulating glucose homeostasis. Increasing the circulating concentration of fatty acids (FAs) induces insulin resistance within hours in humans (7, 52). A similar rapid induction of insulin resistance is induced by palmitate in isolated rodent muscles (3, 4, 44, 61). This fatty acid-induced impairment of insulin sensitivity has been shown to be associated with reduced insulin sensitivity of the insulin signaling pathway, as reflected by the inhibition of insulin stimulated PKB/Akt and AS160 phosphorylation, resulting in an impaired GLUT4 translocation (3, 4, 52). It is widely thought that exposing muscle to FAs increases bioactive intramuscular lipids (diacylglycerol, ceramide and long chain fatty acyl CoAs) which cannot be oxidized away (27, 33). However, there is also evidence that insulin resistance is not always tightly associated with increased intramuscular lipid content (3, 11, 62). Nevertheless, there is good correlation between the ability to oxidize FAs and insulin resistance (3, 4, 12). Therefore, stimulating the intracellular metabolic pathway that regulates FA oxidation appears to protects the muscle cell from lipotoxic effects (3, 16).

In skeletal muscle activation of AMP-activated protein kinase (AMPK) orchestrates a variety of metabolic pathways, including enhanced glucose transport and lipid oxidation (25, 53). Glucose uptake in skeletal muscle is mediated by both insulin-dependent and insulin-independent pathways (50). Activating AMPK by pharmacological approaches (31, 58, 67), muscle contraction and exercise (51, 58, 67) increases glucose uptake in an insulin-independent manner, in both healthy and insulin-resistant muscle (32, 58, 67). Therefore, stimulating the phosphorylation/activation of this enzyme is an attractive therapeutic target to prevent and/or ameliorate insulin resistance, as has been shown recently in Zucker diabetic fatty rats (46).

Activation of AMPK promotes FA oxidation by phosphorylating and deactivating its downstream target acetyl-CoA carboxylase(ACC) which in turn prevents the production of malonyl-CoA (25, 53), thereby
releasing the inhibitory effect exerted by malonyl-CoA on carnitine-palmitoyl-CoA transferase 1 (CPT-1) (25, 53). However, this role of AMPK has recently been questioned (20). Nevertheless, many insulin-sensitizing agents such as, metformin and thiazolidinediones (TZDs), exert their antidiabetic effect, in part, through activation of AMPK(25, 53). Yet, many of TZDs therapies have either limited efficacy or may have undesirable side effects, including body weight gain, enhancement of gastrointestinal problems, and an increased cardiovascular risk (37, 41). Therefore, there is considerable interest in seeking alternative therapeutic approaches, with minimal or no side-effects, to activate AMPK.

Selected herbal preparations have the ability to ameliorate or delay the progression of insulin resistance or type 2 diabetes. For example, *Allium cepa* (garden onion) and *Momordica charantia* (bitter melon) have hypoglycemic effects (13, 17, 24) and improved insulin action in rats fed a high fat diet (17), while the medicinal plant, *Galega officinalis*, led to discovery and synthesis of metformin, the well-known hypoglycemic medication (64). Essential (volatile) oils of herbs and medicinal plants have long been known to have antimicrobial, antifungal and antioxidant properties (10, 60), as well as exerting hypoglycemic effects in animal models of Type 1 diabetes (54, 55), type 2 diabetes (Zucker fatty rats) and insulin resistance (59). Thus, it appears that essential oils offer therapeutic potential for the treatment of insulin resistance, although their mechanism of action remains unknown.

Thujone, a monoterpeno which exists as two stereo isomers (α- and β- thujone), is an ingredient of essential oils of a great many different herbs including *Salvia spp*, *Thuja spp*, *Artemisia spp* and many others (22, 39, 40, 48). Thujone oil has long been used in beverages, food additive and herbal medicine (26, 57), and is thought to be the main constituent of several medicinal herbs that have antidiabetic properties (1, 5, 21, 38). Despite the use of these herbs for the treatment of diabetes, the use of thujone per sé for the treatment of insulin resistance has not been examined. Thus, given a) that thujone is the principal constituent of anti-diabetic herbal medicine and b) that thujone belongs to the family of
essential oils, some of which have therapeutic antidiabetic properties, we speculated that thujone could be a natural occurring agent responsible for improving skeletal muscle insulin sensitivity. Therefore, in the present study, we examined whether thujone is an insulin sensitizing agent that acts by increasing the phosphorylation/activation of AMPK. To examine this question we rapidly induced insulin resistance with a high concentration of palmitate, as we have recently reported (4). Subsequently we attempted to restore insulin sensitivity with thujone, while maintaining high concentrations of palmitate.

**Methods:**

**Materials.** Collagenase type II was purchased from Worthington (Lakewood, NJ). Insulin (Humulin-R) was purchased from Eli-Lilly (Toronto, Ontario). Penicillin and streptomycin were purchased from Invitrogen Corporation (Grand Island, NY). Total and phosphorylated proteins were determined with commercially available antibodies from the following sources anti-phospho-Akt Ser473 and anti-Akt1,2,3 from Santa Cruz Biotechnology (Santa Cruz, CA); anti-phospho-AMPK Thr 172 and anti-AMPK from from Upstate (Lake Placid,NY); anti-phospho-ACC Ser 79 and anti-ACC from cell signaling technology (cell signaling technology (Danvers, MA); anti-GLUT4 from Chemicon International (Temecula, CA USA); anti-AS160 and anti-phospho-AS160 Thr 642 from upstate (Lake Placid,NY); goat-anti-rabbit secondary antibodies from Chemicon International (Temecula, CA); donkey-anti-rabbit secondary antibody from Amersham Biosciences (Oakville, Ontario). Thujone (α and β mixture) and all other reagents were obtained from Sigma-Aldrich (St. Louis, Missouri, USA).

**Animals**

All experiments were approved by the Committee on Animal Care, at the University of Guelph. Male Sprague-Dawley rats (50-60g) bred on site were used in these studies. The animals consumed normal laboratory chow and water ad libitum. At the onset of each experiment rats were anesthetized with Somnotol (6mg/100g body wt, i.p), and the soleus muscles were gently dissected free.
**Muscle Incubation**

Soleus muscles remain viable in vitro, for up to 18 h as we previously described (2, 4). Briefly, in the present study, intact soleus muscles (~20 mg), were pre-incubated (15 min) and then incubated with (2 mM) or without palmitate (control) for 0-12h. At t=0, palmitate-treated muscles were only exposed briefly (5 min) to palmitate and control muscles were also briefly (5 min) incubated without palmitate. All incubations (0-12h) were performed in 10 ml of warmed (30°C), pregassed (95% O₂-5% CO₂) Medium 199, containing 5mM glucose, supplemented with 4% bovine serum albumin V (BSA), penicillin (100 IU/ml) and streptomycin (0.1 mg/ml). To maintain muscle viability, low concentrations of insulin (14.3 µU/ml), which do not alter the rates of glucose transport (2) were also included. Incubation vials were shaken at 110 cycle/min and the gas phase and temperature were maintained at 95%O₂-5%CO₂ and 30°C, respectively. After 6h incubation with or without palmitate, muscles were incubated for an additional 6 hours in fresh media with or without palmitate. During this last 6 hours of incubation some muscles were treated with thujone 0.01mg/ml or with thujone 0.01mg/ml + AMPK inhibitors adenine 9-β-D-arabinofuranoside (Ara) (2.5mM) or compound C (50uM). The doses of thujone (0.01mg/ml) and compound C (50 uM ) were established in pilot studies, as these concentrations provided the optimal effects (data not shown).

**Glucose transport**

[^3]H]-3-O-methyl-D-glucose (3-O-MG) transport was determined according to previously described procedures in our laboratory (4). Briefly, at the end of the specified incubation periods, soleus muscles were incubated (30°C, 30 min, 95% O₂-5% CO₂) in 2 ml of palmitate-free Krebs-Henseleit buffer (8mM glucose, 32 mM mannitol and 0.1% BSA with (20 mU/ml) or without insulin). Subsequently, muscles were washed (2x10 min, 30°C, glucose-free Krebs- Henseleit buffer, 40 mM mannitol, 0.1% BSA, with (20 mU/ml) or without insulin). Glucose transport was then determined in palmitate-free
Krebs-Henseleit buffer (2 ml) supplemented with 0.5 µCi [³H]-3-O-MG, 1.0 µCi [¹⁴C]-mannitol, 32 mM 3-O-MG, 4 mM mannitol, 4 mM pyruvate and 0.1%BSA, in the presence (20 mU/ml) or absence of insulin for 20 min, as previously reported (3, 4). Thereafter, muscles were blotted, weighed and solubilized followed by scintillation counting of muscle extracts.

**Palmitate oxidation**

To determine the rate of FAs oxidation, our previously described method was used (4). Briefly, at the end of the incubation periods (0, 6, and 12h), isolated soleus muscles that had been incubated with and without palmitate were transferred to other glass vials containing 2 ml of pregassed (95% O₂-5% CO₂) Medium 199 supplemented with 4% BSA and palmitate (2 mM, 0.5 µCi/ml [¹⁴C]-palmitate). Palmitate oxidation occurred at 30°C for 40 min, and the ¹⁴CO₂ released was captured in a benzothium hydroxide trap (400 µl, 1.0 M). In addition, at the end of the 40-min incubation period, dissolved CO₂ was released by adding sulfuric acid (1.0 ml, 1 M) to a 1.0-ml aliquot of the incubating medium and capturing the ¹⁴CO₂ in a benzothium hydroxide trap. Finally, water-soluble ¹⁴C-labeled intermediates were extracted from muscles homogenized after their incubation (4). After scintillation counting, the palmitate oxidation rate was determined as we have done previously (4), by summing the three sources of [¹⁴C]palmitate.

**Plasma Membrane Preparation**

The plasmalemmal content of GLUT4 protein was determined in soleus muscles incubated in Medium 199 for 0, 6 and 12 h with or without palmitate. At these time points, the muscles were treated with insulin (20 mU/ml) for 70 min to mimic the time course in the foregoing glucose transport experiments. Thereafter giant vesicles were prepared in which plasma membrane content of GLUT4 was measured, as we have previously reported (3, 4). To obtain sufficient plasma membrane, 10 incubated soleii were pooled for each independent experiment. In total 40 such independent preparations were performed.
Giant vesicle plasma membranes were obtained as we have previously reported in detail (8, 9). Briefly, soleus muscles were cut into thin layers (~1-3mm thick) with scalpel. The scored muscles were then incubated for 75min at 34°C in 140 mM KCl-10 mM MOPS (pH 7.4), collagenase (150 U/ml), and aprotinin (1 mg/ml). Thereafter, the incubating medium was collected and the remaining muscle debris as washed with 10 mM EDTA in KCl/MOPS until 7ml had been collected. Percoll (final concentration 16%) and aprotinin(1 mg/ml) were added to the collected medium. The resulting mixture was placed at the bottom of a density gradient consisting of a 3-ml middle layer of 4% Nycodenz (wt/vol) and a 1-ml KCl-MOPS upper layer. The samples were spun at 60 g for 45 min at room temperature. After centrifugation, the vesicles were harvested from the interface of the two upper solutions and centrifuged at 12,000 x g for 5 min. The supernatant fraction was aspirated and the resulting pellet was resuspended in KCl/MOPS. Vesicles were stored at -80°C for subsequent analysis.

Protein Analysis

The expression of selected proteins was determined in soleus muscle incubated in Medium 199 for 0, 6 and 12 h without or with palmitate. Thereafter, muscles were rapidly blotted, frozen in liquid nitrogen, and stored at –80°C until analyzed. To measure the phosphorylation of AS160, muscles were incubated for the various experiments as described above, followed by incubation with insulin (20 mU/ml) for 10 min, the time in which maximal phosphorylation was observed (4). Thereafter, the muscles were rapidly blotted, frozen and stored at -80°C for later analysis.

Muscle Protein Extraction and Western Blotting

For whole muscle protein determination, frozen soleus muscle was homogenized in 2 ml of buffer. Muscle homogenate and plasma membrane protein concentrations were determined using the bicinchoninic acid assay. Proteins were separated using SDS-polyacrylamide gel electrophoresis and were detected using Western blotting. We (4, 6) have reported these procedures previously.
Statistics

Data were analyzed using two-way analyses of variance. For some experiments, the data were analyzed with a one-way analysis of variance, when this was warranted, and when appropriate, a Fisher’s LSD post-hoc analysis was used. All data are reported as mean ± SEM.

Results

Effect of thujone on insulin-stimulated glucose transport:

In the absence of palmitate, basal glucose transport was not altered during the 12h incubation period ($P > 0.05$; Fig. 1). In the palmitate-treated muscles, the basal glucose transport was reduced slightly by 0.25 µmol/g/20 min) within the first 6 h ($P < 0.05$; Fig. 1). Thereafter (6–12h), basal glucose transport remained unaltered ($P > 0.05$; Fig. 1).

In control muscle, insulin-stimulated glucose transport was not altered over the 12h incubation period ($P > 0.05$; Fig.1). In contrast, palmitate induced a progressive reduction in insulin-stimulated 3-OMG transport over 12h. After 6, and 12h, glucose transport was reduced by 39% ($P < 0.05$), and 71% ($P < 0.05$), respectively.

Treatment with thujone (0.01mg/ml) for 6 h restored insulin-stimulated glucose transport to normal despite the continued presence of palmitate (2 mM) (Fig. 1). In palmitate-treated muscles, the thujone-induced increase in basal glucose transport ($\Delta = + 0.31$ µmol/g/20 min) cannot account for the much larger, thujone-induced increase in insulin stimulated glucose transport ($\Delta = 1.47$ umol/g/20 min) (Fig. 1).

Effect of thujone on insulin-stimulated plasmalemmal GLUT-4:

The expression of GLUT4 protein was not altered with any of the experimental treatments (data not
shown). In control muscles insulin stimulation induced similar increases in the levels of plasmalemmal GLUT4 at 0, 6 and 12 h ($P > 0.05$; Fig. 2A). In contrast, in palmitate-treated muscles, the insulin-stimulated GLUT4 translocation was markedly reduced by 40% within the first 6h ($P < 0.05$, Fig 2A). Thereafter, palmitate treatment did not further reduce the insulin-stimulated GLUT4 content at the plasma membrane at 12h ($P > 0.05$; Fig.2A).

In control muscles treated with thujone, the insulin-stimulated appearance of GLUT4 at the plasma membrane was increased (+27%, $P < 0.05$, Fig 2A). In muscles treated with palmitate for 12 h, the addition of thujone for the last 6h increased insulin-stimulated GLUT4 appearance at the plasma membrane, although not to the levels in control muscle. Specifically, the net increase in the insulin-stimulated plasma membrane GLUT4 was similar in the thujone-treated control and thujone + palmitate-treated muscles. However, the insulin-stimulated plasma membrane GLUT4 was 22% ($P < 0.05$) lower in the thujone + palmitate-treated muscles than in untreated control muscles (Fig. 2A).

*Effect of thujone on AS160 phosphorylation:*

The protein expression of AS160 was not altered during (0-12h) incubation period in either the control or palmitate-treated muscles, or in the thujone treated muscles (data not shown). In control muscles, insulin-stimulated AS160 phosphorylation was not altered ($P > 0.05$; Fig. 2B). In contrast, palmitate treatment reduced insulin stimulated AS160 phosphorylation by 26% after 6h ($P < 0.05$; Fig. 2B), and AS160 remained at this reduced level of activation until 12h (Fig. 2B). In control muscles treated with thujone, the insulin-stimulated AS160 phosphorylation did not change (Fig. 2B). However, in palmitate-treated muscles thujone fully rescued the insulin-stimulated phosphorylation of AS160 ($P < 0.05$, Fig. 2B), despite the continued presence of palmitate (2 mM).
Effect of thujone on palmitate oxidation:

The rate of palmitate oxidation was not altered in control muscles \((P > 0.05; \text{Fig. 3})\). In contrast, in the palmitate-treated muscles, palmitate oxidation was markedly decreased by 40% at 6 h \((P < 0.05; \text{Fig.3})\) and remained reduced (-50%) up to 12h of incubation. In palmitate-treated muscles, thujone increased the palmitate oxidation rate \((P<0.05)\) to the levels observed in the untreated control muscles \((\text{Fig.3})\).

Effect of thujone on AMPK phosphorylation:

The total expression of AMPK did not change during 12h incubation in control, palmitate- or thujone-treated muscles (data not shown). Incubating soleus muscles without palmitate \((0-12h)\) increased AMPK phosphorylation \((+30\% \text{ at } 6h; +31 \% \text{ at } 12h)\) \((P<0.05, \text{Fig 4A})\). This was also observed in the palmitate treated muscles, although compared to the control muscle the increases at 6h (+50%) and at 12 h (+51%) were somewhat greater \((P<0.05)\). In control muscles, AMPK phosphorylation was not increased further by thujone treatment. In contrast, in the palmitate-treated muscles, thujone treatment increased AMPK phosphorylation an additional 48% beyond the increase observed in palmitate-treated muscles at 6 h \((P<0.05, \text{Fig 4A})\).

Effect of thujone on ACC phosphorylation:

The total expression of ACC did not change during 12h incubation in control, palmitate-, or thujone-treated muscles (data not shown). Incubating muscles without palmitate \((0-12h)\) increased ACC phosphorylation \((+26\% \text{ at } 6h; +21\% \text{ at } 12h)\) \((P<0.05, \text{Fig 4B})\). This also occurred in the palmitate-treated muscles, although ACC phosphorylations were greater at 6h (+48%) and at 12 h (+44%) \((P<0.05)\). Thujone treatment did not alter ACC phosphorylation either in control or palmitate-treated muscles \((P>0.05, \text{Fig 4B})\).
**Effect of Ara and compound C on AMPK and ACC phosphorylation:**

To establish whether the effect of thujone on insulin action was attributable to AMPK activation, we performed independent experiments with inhibitors of AMPK phosphorylation, namely Ara (2.5mM) and compound C (50 µM). Each of these reduced AMPK phosphorylation (Ara -32%, -56% and compound C -42%, -63%) in control and palmitate-treated muscles respectively (P<0.05; Fig. 5A). This reduction was not prevented by thujone. Concurrently, ACC phosphorylation was also reduced by Ara (-22%) or compound C (-18%) in the presence of both palmitate and thujone (P <0.05; Fig. 5B).

**Effect of Ara or compound C on restorative effects of thujone on glucose transport, insulin signaling and palmitate oxidation**

In palmitate-treated muscles both compound C and Ara prevented the thujone-induced recovery of insulin-stimulated glucose transport (P<0.05; Fig. 6A). These inhibitors however exerted no effect on insulin-stimulated glucose transport in control muscles (P>0.05; Fig. 6A). Similar effects were observed with respect to AS160 phosphorylation, namely, compound C and Ara inhibited AS160 phosphorylation only in the palmitate-treated muscles (P<0.05; Fig. 6B), not in control muscles (P>0.05; Fig. 6B).

The addition of AMPK inhibitors Ara or compound C largely failed to inhibit the restorative effects of thujone on the rate of palmitate oxidation. Specifically, the thujone-mediated increases in palmitate oxidation were only inhibited by 20% (Ara) and 18% (compound C) (P<0.05; Fig. 6C).

**Discussion**

Thujone is a monoterpane present in essential oils of several medicinal plants and herbs that have antidiabetic properties (1, 5, 21, 38). Therefore, we examined whether thujone was able to rescue palmitate-induced insulin resistance in skeletal muscle. Our study has provided novel information on the thereapeutic effects of thujone on insulin resistance, and on its mechanism of action. Specifically, we
found that 1) thujone completely recovered insulin sensitivity of insulin resistant muscles, despite the continued presence of palmitate. This marked improvement in insulin action was closely associated with the complete restoration of 2) AS160 phosphorylation and 3) palmitate oxidation, but 4) only a partial restoration of insulin-stimulated GLUT4 translocation to the plasma membrane. Although 5) thujone activated AMPK, only 6) some of the restorative effects of thujone were associated with the concurrent activation of AMPK, namely insulin-stimulated AS160 phosphorylation and glucose transport, but not palmitate oxidation. These studies have shown that thujone, a naturally occurring plant product, can rescue palmitate-induced insulin resistance in muscle.

**GLUT-4 mediated changes in glucose transport induced by palmitate and by thujone**

It is now well known that the impaired recruitment of GLUT4 to cell surface plays an essential role in the induction of insulin resistance (35, 65). In agreement with our previous work (4) and others (47, 56, 66), the present study indicates that impairment in insulin-stimulated GLUT4 translocation is an essential mechanism by which palmitate induced insulin resistance. However, it may not be the only mechanism. For example, despite the further reduction in insulin-stimulated glucose transport from 6-12 h in palmitate-treated muscles, insulin-stimulated GLUT4 translocation was not further reduced during this time period. This may reflect a reduction in the intrinsic activity of plasmalemmal GLUT4, as has been suggested previously (4). Alternatively, the discord between insulin-stimulated glucose transport and plasma membrane GLUT4 might reflect the fact that glucose transport was measured in intact muscle in which GLUT4 is present at both the plasma membrane and t-tubules, while cell surface GLUT4 was measured only on the plasma membrane of giant vesicles, which are devoid of t-tubules. While it would be ideal to measure glucose transport in giant vesicles rather than in intact muscle, for practical reasons it was not possible to measure glucose transport in the giant vesicles as this would have required pooling of 50 soleii for a single determination. However, since it has been shown that insulin stimulated GLUT4 translocation to the plasma membrane and to the t-tubules is similar (36), it is not
possible to completely discount the notion that a change in GLUT4 intrinsic activity was also involved in altering insulin-stimulated glucose transport.

Thujone rescued insulin stimulated-glucose transport, despite maintaining high concentration of palmitate. These thujone-induced improvements were not attributable to changes in the basal rates of glucose transport, as these were only altered minimally by thujone. The thujone-induced improvements in insulin-stimulated glucose transport and GLUT4 translocation did not completely parallel each other. This may suggest that the thujone treatment exerted an insulin-sensitizing effect in muscle by other mechanism(s) in addition to altered GLUT4 translocation, possibly an improved intrinsic activity of GLUT4. This interpretation, as we have noted above, is complicated by the fact that glucose transport was measured in the intact muscle, while plasma membrane GLUT4 was determined in giant vesicles. Nevertheless, we suggest that the thujone treatment rescued insulin resistance by a) partially restoring the insulin-stimulated GLUT4 translocation and b) possibly by also improving the intrinsic activity of plasmalemmal GLUT4.

*AS160 phosphorylation restoration does not fully restore GLUT4 translocatin:*

AS160, a downstream target in the insulin signaling pathway, plays a critical role in insulin-stimulated GLUT4 translocation, and hence in glucose uptake (15, 49). In agreement with previous work (3), induction of insulin resistance by palmitate treatment, reduced insulin-stimulated AS160 phosphorylation, and this was associated with the reduction in plasma membrane content of GLUT4 at 6h, and their continued reduction to the same levels until 12 h. Thus, the impaired insulin–stimulated GLUT4 translocation appears to be due to the reduced activation of AS160 in palmitate treated muscle. In contrast, while thujone fully restored insulin-stimulated AS160 phosphorylation this did not fully
restore insulin-stimulated plasma membrane GLUT4 content. This implies that other signals may also be required to fully restore GLUT4 translocation.

*Effect of thujone on palmitate oxidation and AMPK/ACC phosphorylation:*

Numerous studies have shown that reductions in FA oxidation are associated with reduced insulin sensitivity in skeletal muscle, as this can lead to an intramuscular accumulation of bioactive lipids that interfere with insulin signaling (4, 34, 63). As in our previous work (4), we again observed that prolonged (0-12h) incubation with palmitate reduced the oxidation rate of this substrate. Importantly, we demonstrate for the first time, that thujone fully rescued palmitate oxidation, despite the maintenance of high concentrations of palmitate.

It has been widely thought that AMPK activation in skeletal muscles stimulates FA oxidation via the inhibition of ACC activity(14, 29, 43). However, the necessity for AMPK activation to stimulate FA oxidation has recently been called into question by several groups (20, 23, 45) as well as in the present study. For example, incubating muscle with or without palmitate increased AMPK and ACC phosphorylations, although, for unknown reasons, this effect was somewhat greater in the palmitate-treated muscles. Yet, despite the increase in AMPK phosphorylation, palmitate oxidation was unaltered in control muscles and was suppressed by 40-50% in the palmitate-treated muscles (present study and (3)). Similar observations have also been reported in palmitate-treated L6 myotubes (45). On the other hand, in palmitate + thujone-treated muscles, AMPK phosphorylation was associated with the increase in palmitate oxidation, although the basis for this thujone-mediated effect is not known. Nevertheless, in the present study and others (20, 23, 45) the relationship between AMPK phosphorylation and fatty acid oxidation has been questioned, especially as ACC phosphorylation can be maintained, despite a lack of AMPKα2 activation in muscle (20), heart (18) or hepatocytes (30). In addition, in the present study the thujone-induced palmitate oxidation was only slightly reduced, when AMPK phosphorylation was
inhibited. This observation as well as others (18, 20, 23, 30, 42, 45) suggest that palmitate oxidation may be regulated in an AMPK-independent manner, by as yet unknown kinases that remain to be identified.

Fatty acid oxidation and insulin sensitivity:

Many studies (3, 4, 19, 28, 34) have found a positive association between the increase in palmitate oxidation and insulin-stimulated glucose transport. In the present study, a similar association appeared to be present, as the palmitate-induced reduction and the thujone-stimulated rescue of insulin-stimulated glucose transport were accompanied by concurrent changes in fatty acid oxidation. However, it seems unlikely that thujone-stimulated improvement in palmitate oxidation is the direct cause of improved insulin sensitivity, for the following reason: AMPK inhibitors only inhibited thujone-stimulated fatty acid oxidation slightly while completely blocking the thujone-mediated rescue of insulin-stimulated glucose transport. We acknowledge that we cannot entirely rule out the possibility that the “AMPK sensitive” component of fatty acid oxidation was still present and was able to reverse the effects of palmitate-induced insulin resistance. Nevertheless, on balance, our observations would seem to suggest that the restorative action of thujone on insulin-stimulated glucose transport is largely attributed to the activation of AMPK, rather than an increase in fatty acid oxidation.

Conclusion

The present study has shown that thujone, a major component of several medicinal herbs, had an effective insulin sensitizing action, which rescues insulin-stimulated glucose transport by restoring insulin-stimulated AS160 phosphorylation, which partly normalized insulin-stimulated GLUT4 translocation to cell surface. In addition it appears that thujone also enhanced the GLUT4 intrinsic activity. Additionally, thujone completely restore palmitate oxidation. However this restoration in the rate of palmitate oxidation could not easily be attributed to the activation of AMPK, as inhibition of AMPK phosphorylation provoked only a partial inhibition in palmitate oxidation.
**Perspective and significance**

Selected herbal preparations have the ability to ameliorate or delay the progression of insulin resistance or type 2 diabetes, as these can have hypoglycemic effects. While a similar therapeutic potential is also provided by essential (volatile) oils of herbs and medicinal plants, their mechanism of action remains unknown. Our work has identified thujone, a naturally occurring compound and the main constituent of several medicinal herbs that have antidiabetic properties, as a compound that can improve insulin sensitivity in skeletal muscle. With further study as to its exact mechanism of action and its safety in humans, thujone may in the long term prove to be a low cost therapeutic agent. Thus our present findings may be significant in the development of novel therapies.
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References:


List of Figures

Fig.1. Insulin-stimulated glucose transport in control and palmitate (2mM) treated muscles (0-12 h), and in muscles that were treated with thujone (0.01mg/ml) for 6 h (6-12h), while maintaining high concentrations of palmitate (2mM).

Data are presented as mean ± SEM

Insulin was only provided (70 min) at the end of each incubation period.

N=8-10 soleii per data point.

* P<0.05 palmitate-treated muscles t=6h or 12h vs control muscles t =6h or 12h

** P<0.05 palmitate-treated muscles t=6h or 12h vs t =0

¶ P<0.05 palmitate-treated muscles t =12h vs t=6 h

+ P<0.05 palmitate-treated muscles + thujone treatment at t=12h vs palmitate-treated muscles at t=12h

++ P<0.05 control muscles + thujone treatment at t=12h vs control muscles at t=12h

Fig.2. Insulin-stimulated plasma membrane GLUT4 (A) and AS160 phosphorylation (B) in control and palmitate (2mM) treated muscles (0-12 h), and in muscles that were treated with thujone (0.01mg/ml) for 6 h (6-12h), while maintaining high concentrations of palmitate (2mM).

Data are presented as mean ± SEM

To measure plasma membrane GLUT4, insulin was only provided (70 min) at the end of each incubation period.
To measure AS160 phosphorylation, insulin was provided for 10 min at the end of each incubation period.

N=6-7 independent experiments for each data point; to obtain sufficient plasma membrane, 10 soleii were pooled for each independent experiment at each time point.

Equal quantities of protein were loaded for each muscle at each time point and loading was verified with Ponceau staining.

* P<0.05 palmitate-treated muscles t=6h or 12h vs control muscles t=6h or 12h

** P<0.05 palmitate-treated muscles t=6h or 12h vs t=0

+ P<0.05 pamitate-treated muscles + thujone at t=12h vs palmitate-treated muscles at t=12h

++ P<0.05 control muscles + thujone treatment at t=12h vs control muscles at t=12h

**Fig.3.** Palmitate oxidation in control and palmitate (2mM) treated muscles (0-12 h), and in muscles that were treated with thujone (0.01mg/ml) while maintaining high concentrations of palmitate (2mM). Data are presented as means ± SEM

N=4-6 soleii per data point

* P<0.05 palmitate-treated muscles t=6h or 12h vs control muscles t=6h or t=12h

** P<0.05 palmitate-treated muscles t=6h or 12h vs t=0

¶ P<0.05 palmitate-treated muscles t=12h vs t=6h

+ P<0.05 palmitate-treated muscles + thujone at t=12h vs palmitate-treated muscles at t=12h

**Fig.4.** AMPK (A) and ACC (B) phosphorylations in control and palmitate (2mM) treated muscles (0-18 h), and in muscles that were treated with thujone (0.01mg/ml) for 6 h (12-18h) while maintaining high concentrations of palmitate (2mM).

Data are presented as means ± SEM

N=4-8 soleii per data point
Equal quantities of protein were loaded for each muscle at each time point and loading was verified with Ponceau staining.

* P<0.05 palmitate-treated muscles t=6h or 12h vs control muscles t =6h or t=12h

** P<0.05 palmitate-treated muscles t=6h or 12h vs t =0

+ P<0.05 palmitate-treated muscles + thujone at t=12h vs palmitate-treated muscles at t=12h

**Fig. 5.** Effect of compound C (Cc) and Ara on AMPK (A) and ACC (B) phosphorylations in muscles that were treated with thujone (T) (0.01mg/ml) and with and without palmitate. Data are presented as means ± SEM

N=4-8 soleii per data point

Equal quantities of protein were loaded for each muscle at each time point and loading was verified with Ponceau staining.

*P<0.05 thujone (T) + compound C (Cc) at t=12h vs control (C) muscles and thujone (T) treated muscles at t=12h

**P<0.05 thujone (T) + Ara at t=12h vs control (C) muscles and thujone (T) treated muscles at t=12h

+ P<0.05 palmitate-treated muscles + thujone (T) at t=12h vs control muscles at t=12

++ P<0.05 thujone (T) + compound C (Cc) at 12h vs thujone (T) treated muscle at t= 12h

**Fig. 6.** Effect of compound C (Cc) and Ara on insulin-stimulated glucose transport (A), AS160 phosphorylation (B) and palmitate oxidation (C) in muscles that were treated with thujone (T) (0.01mg/ml) and with and without palmitate. All measurements were performed at 12h.

Data are presented as means ± SEM

N=4-10 soleii per data point

Equal quantities of protein were loaded for each muscle in panel B, and loading was verified with Ponceau staining.
* P<0.05 thujone (T) + compound C (Cc) at t=12h vs thujone (T) treated muscles at t=12h

**P<0.05 thujone (T) + Ara at t=12h vs thujone (T) treated muscles at t=12h

+ P<0.05 thujone (T) + compound C (Cc) at t=12h vs control (C) muscles at t=12h

++ P<0.05 thujone (T) + Ara at t=12h vs control (C) muscles at t=12h
Fig. 1
Fig. 2
Fig. 3
Fig. 4
Fig. 5
Fig. 6