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ORIGINAL CONTRIBUTION

Indu Singh
Andrew L. Carey
Nadine Watson
Mark A. Febbraio
John A. Hawley

# Oxidative stress-induced insulin resistance in skeletal muscle cells is ameliorated by gamma-tocopherol treatment

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I. Singh and A.L. Carey contributed equally.

I. Singh · A.L. Carey
J.A. Hawley, PhD (✉)
Exercise Metabolism Group
School of Medical Sciences
RMIT University, PO Box 71
Bundoora (VIC) 3083, Australia
E-Mail: john.hawley@rmit.edu.au

N. Watson · M.A. Febbraio
Cellular and Molecular Metabolism Laboratory
Baker Heart Research Institute
Melbourne (VIC), Australia

**Abstract** *Background* Oxidative stress-induced reactive oxygen species are associated with the clinical manifestation of insulin resistance. Evidence suggests that antioxidant treatment may reduce this incidence. *Aim of the study* This study determined whether glucose oxidase (GO)-induced insulin resistance in cultured skeletal muscle cells could be ameliorated by pre-treatment with gamma-tocopherol (GT). *Methods* Insulin sensitivity in L6 myotubes was assessed by 2-deoxy-D-[$^3$H]-glucose uptake. The phosphorylation of distal insulin signaling proteins Akt and the Akt substrate AS160 were determined by western blot. *Results* One hour treatment with 100 mU/ml GO decreased insulin-stimulated glucose uptake ($P < 0.001$). Pre-treatment with GT either partially (100 μM) or completely (200 μM) restored insulin-stimulated glucose uptake in cells after GO-induced insulin resistance. GO-induced oxidative stress did not impair insulin stimulated phosphorylation of Akt or AS160, but 200 μM GT increased insulin-stimulated phosphorylation of these key signaling proteins ($P < 0.05$). *Conclusions* High-dose (200 μM) GT treatment ameliorated oxidative stress-induced insulin resistance in cultured rat L6 skeletal muscle cells.

**Key words** glucose oxidase – L6 myotubes – Akt – AS160 – glucose transport – antioxidants

## Introduction

There is accumulating evidence that the generation of reactive oxygen species (ROS) leads to increased oxidative stress and is a precursor to a number of chronic conditions including type-2 diabetes and cardiovascular disease [33]. Antioxidant agents have been proposed to arrest some of the deleterious effects of ROS [1, 17] either by donating hydrogen to free radicals or by accepting free radicals and therefore neutralizing their effect. Gamma-tocopherol (GT), an isoform of Vitamin E present in food has been shown to have potent antioxidant effects [33]. GT inhibits platelet aggregation to a greater extent than alpha-tocopherol, as platelets isolated from humans supplemented with a mixed-tocopherol preparation exhibited reduced aggregation after mixed-tocopherol compared to alpha-tocopherol supplementation [20]. In addition, we have recently demonstrated that pure GT inhibited platelet aggregation and improved lipid profile in normal healthy subjects, thereby playing a potential role in prevention of thrombosis [28].

Type-2 diabetes is characterized by insulin resistance in various tissues; in particular, skeletal muscle,

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the primary site for insulin-stimulated glucose disposal [2]. While there is evidence that oxidative stress leads to insulin resistance [6, 17], tissue damage and pathogenesis of late diabetic complications [25], the exact mechanisms responsible remain unclear. In skeletal muscle cells, insulin-stimulated uptake of glucose depends on a number of signaling components, culminating in the translocation of vesicles containing the glucose transporter GLUT4 to the cell surface [16]. The effects of ROS on the insulin signaling cascade are equivocal [10, 13, 14, 26], but the results of several studies demonstrate that ROS impairs insulin-mediated glucose uptake and storage [4, 10, 18, 27, 32], via disruption at signaling control points such as glycogen synthase kinase-3 [4] Akt phosphorylation and/or actin remodeling [14]. While it has been proposed that Vitamin E supplementation might prevent ROS-induced impairment of insulin signaling [7], and improve biomarkers of metabolic syndrome [3] studies of the effects of antioxidant treatment on insulin signaling and simultaneous functional measures of glucose transport are lacking. If ROS are involved in causing damage to cellular signaling machinery, antioxidant treatment may be able to alleviate or prevent such an occurrence. Accordingly, the primary purpose of the current investigation was to determine whether GT, which we have previously demonstrated to have potent antioxidant properties [28], could ameliorate the deleterious effects of oxidative stress on insulin sensitivity. In addition, we also investigated the effects of GT treatment on the most distal signaling protein identified in signaling to GLUT4 vesicles for insulin-stimulated translocation to the cell surface, the Akt substrate AS160.

## Materials and methods

### Cell culture

L6 skeletal myoblasts (American Type Cell Collection, VA, USA) were cultured using aseptic techniques in 75 $cm^2$ flasks containing growth media, which consisted of DMEM supplemented with 10% FBS, 1% penicillin/streptomycin (v:v) at 37°C in 5% $CO_2$/95%$O_2$ in humidified air. Media was replaced daily with pre-warmed media (37°C) until the cells were 60–70% confluent. Cells were washed twice with pre-warmed PBS then detached with 2 ml of 0.25% trypsin for 3–4 min with intermittent shaking. Once the cells were completely detached the total number of cells were counted then seeded into six well plates at 7,000 cells per $cm^2$. Cells were grown to 60–70% confluency in α-modified essential medium (αMEM) +10% FBS before changing to differentiation media (αMEM +2% FBS) to allow myoblasts to differentiate into myotubes. Experimental treatments were conducted after 2 days, by which time nearly all of the myoblasts had fused to form myotubes. Cells were incubated with GT for 24 h (with concentrations indicated) and oxidative stress was induced by incubation with 100 mU/ml glucose oxidase (GO) during the final 1 h of this 24 h period. Similar doses and durations of GO treatment in skeletal myotubes or isolated skeletal muscle have been shown repeatedly to induce >50-fold increases in cellular $H_2O_2$ concentrations without affecting cell viability [4, 18, 21, 31].

### Glucose uptake

For determination of 2-deoxy-D-[$^3$H]-glucose uptake, myotubes were incubated in αMEM without glucose with 0.1% FBS for 4 h. Cells were washed twice with warm PBS (containing 0.1% FBS) and equilibrated in 2 ml αMEM (without glucose) for 30 min followed by incubation with or without 100 nM insulin for 30 min, before 800 µl of radioactive media (1 µCi/ml 2-deoxy-D-($^3$H)-glucose, 10 µM 2-deoxy-D-glucose in αMEM, 0.1% FBS) was added to each well. After 30 min media was aspirated and the assay stopped by washing cells twice in ice-cold PBS. Cells were then lyzed in 1 ml of 0.3 M NaOH and 800 µl of the lysate in 4 ml of scintillation fluid was counted via liquid scintillation.

### Western blotting

L6 myotubes were grown, differentiated and treated as described under cell culture above. Lysis buffer [20 mM HEPES (pH 7.4), 2 mM EDTA, 50 mM NaF, 5 mM $Na_4P_2O_7$, 1% NP40 and phosphatase and protease inhibitor cocktails] was added and cells rapidly scraped then transferred to a 1.5 ml tube and rapidly frozen in liquid nitrogen for later analysis. Subsequently, cell culture lysates were spun at 16,000*g* for 5 min, then 5 µl of the supernatant taken and diluted 1:25 with water, before the protein concentration was determined using a commercially available kit (BCA™ Protein Assay Kit, Pierce, USA) using absorbance spectrophotometry. Absorbance of standards and samples was determined at 560 nm on a spectrophotometer and the protein content calculated from the linear regression.

Proteins solubilized in Lamelli's buffer (pH 6.8 consisting of 40% glycerol, 8.2% sodium dodecyl sulfate (SDS), 0.5 M Tris–HCl, 40 mM dithiotrietol plus bromophenol blue) were added to 40 µg of sample, heated for 5 min at 85°C, and proteins resolved by SDS–PAGE using pre-cast polyacrylamide gradient gels and commercially available electrophoresis reagents (Invitrogen, VIC, Australia). A molec-

ular weight protein standard (Biorad, NSW, Australia), and solubilized protein samples were loaded into individual wells and run at 150 V until proteins had sufficiently migrated through the gel.

Separated proteins were then transferred to a PVDF membrane using pre-developed reagents and standard transfer sandwich apparatus (Invitrogen, VIC, Australia). Proteins were transferred at a constant voltage of 20 V for 100 (Akt) or 180 (AS160) min. After the transfer, the membrane was washed with Tris-buffered saline with Tween (TBST; 20 mM Tris, 140 mM NaCl, 0.05% Tween 20, pH 7.6). The membrane was blocked with 5% skim milk powder (w/v) in TBST on a rocker at room temperature for 1 h. After blocking, membranes were washed with TBST and incubated with primary antibodies (diluted 1:1,000 in TBST + 1% BSA) specific for phospho-Akt-Ser473, $\beta$-actin, Phospho-Akt-Substrate, total Akt (Cell Signaling Technology: Genesearch, QLD, Australia), phosphor-AMPK-Thr172 and total AMPK-$\alpha$1/2 (kind gifts from Prof Bruce Kemp) overnight on a rocker at 4°C. Membranes were washed three times with TBST then incubated with secondary antibody (1:5,000 anti-rabbit antibodies in TBST) for 1 h at room temperature. The membrane was then washed (6 × 10 min) with TBST and immunoreactive bands detected with 2 ml of chemiluminescence reagent (Pierce: Quantum Scientific, QLD, Australia) and exposed using the Chemidoc EQ system (Biorad, NSW, Australia). Proteins were quantified using Quantity One software version 9 (Biorad, NSW, Australia).

### Statistical analysis

Data were analyzed using 2-way ANOVA, and $P$ values of <0.05 were considered significant. Results are presented as mean ± SEM.

## Results

Insulin treatment significantly increased glucose transport by ~40% above vehicle ($P < 0.001$). One hour treatment with 100 mU/ml GO significantly decreased insulin-stimulated glucose transport ($P < 0.001$), whereas pre-treatment with 100 μM GT partially protected cells from the effect of GO, and 200 μM GT restored ($P > 0.05$) insulin-stimulated glucose transport to similar levels as non-GO treated cells (Fig. 1).

To assess whether the protective effect of GT on basal and insulin-stimulated glucose transport after induction of oxidative stress was due to improved signaling from the insulin receptor to GLUT4 vesicles, the most distal of the well-characterized proteins in the insulin-GLUT4 signaling cascade were measured; we assessed the phosphorylation of Akt and AS160 on

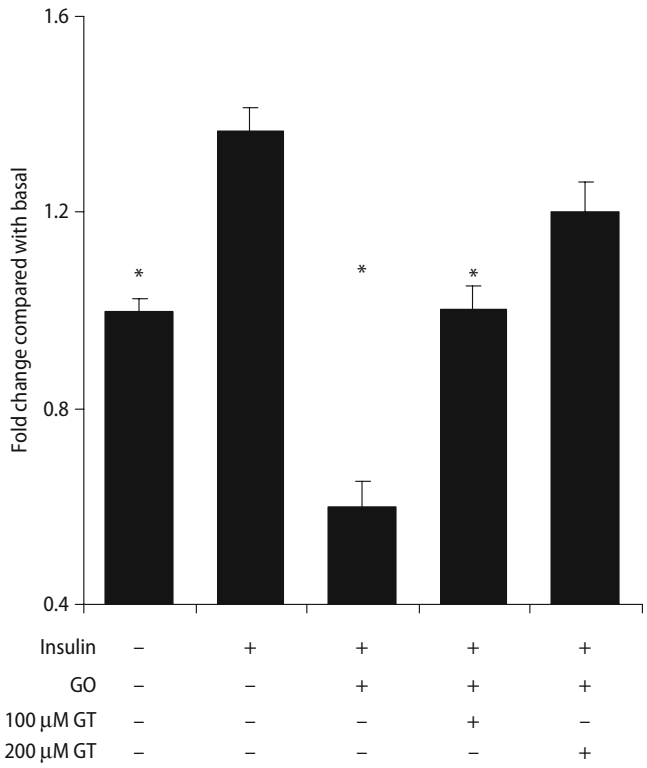


**Fig. 1** 2-Deoxyglucose uptake in L6 myotubes that were differentiated as stated in "Materials and methods". Cells were treated with 100 or 200 μM gamma-tocopherol (*GT*) for 24 h, 100 mU/ml GO for 1 h and 100 nM insulin for 30 min ($n = 8$–11). *Asterisks* significantly lower than treatment with insulin alone ($P < 0.001$)

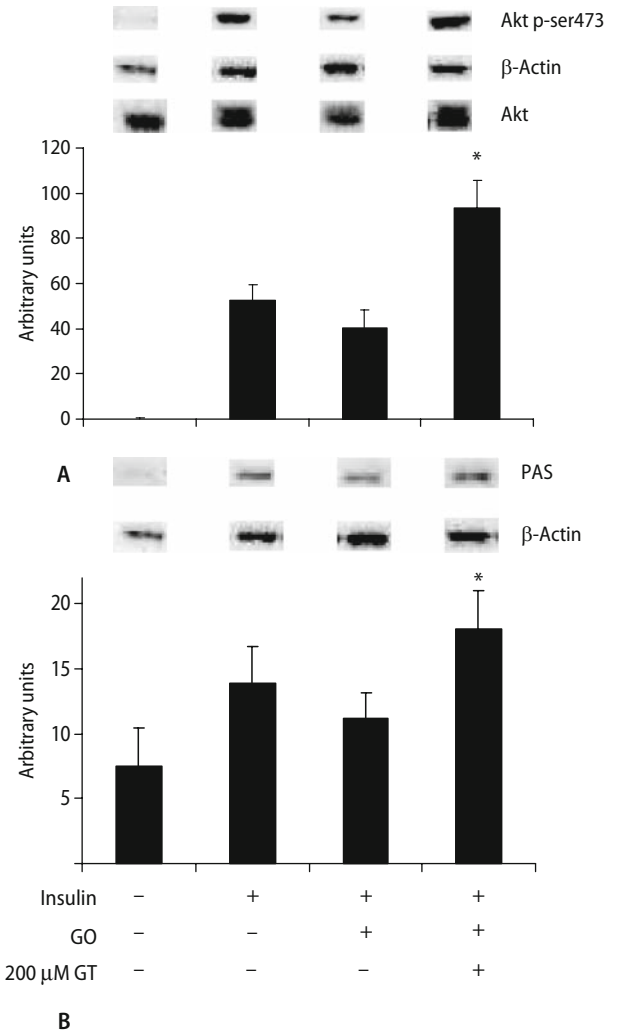


**Fig. 2** Phosphorylation of Akt serine-473 (**a**) and AS160 (**b**) relative to total β-actin in L6 myotubes that were differentiated as described in "Materials and methods". Cells were treated with 200 μM GT for 24 h, 100 mU/ml GO for 1 h and 100 nM insulin for 10 min ($n = 6$). *PAS* phospho-Akt substrate. *Asterisks* significantly increased above all other conditions ($P < 0.05$)

amino-acid residues that are important for insulin signaling to GLUT4. We found that GO did not impair insulin-stimulated phosphorylation of Akt or AS160, but 200 μM GT increased insulin-stimulated phosphorylation of both ($P < 0.05$) above insulin treatment alone, and might partly explain the restoration of glucose transport in this condition (Fig. 2). In addition, since phosphorylation of neither Akt nor AS160 could completely explain the effects of GT on glucose transport, we measured phosphorylation of AMPK to determine whether activation of this stress-activated kinase could explain the changes seen. Phosphorylation of AMPK at Thr172, representative of an increase in activity [11] that would increase glucose transport [12], was not significantly altered by GT treatment (data not shown).

## Discussion

In the present study we investigated the effects of GT on insulin-stimulated glucose transport and selected components of the insulin signaling cascade in cultured rat L6 myotubes exposed to a $H_2O_2$ generating system. While GO treatment significantly reduced insulin-stimulated glucose transport, this reduction did not appear to be due to an impairment of insulin signaling, reflected by unaltered phosphorylations states of the distal insulin signaling proteins Akt and AS160. In contrast, treatment with 200 μM GT ameliorated the GO-induced impairment in insulin-stimulated glucose transport and this coincided with significant increases in the insulin-stimulated phosphorylation of both Akt and AS160. Indeed, the phosphorylation states of these signaling proteins were greater after the high dose of GT than observed when treated with insulin alone (regardless of whether or not cells were co-incubated with GO).

Our finding that GO-mediated oxidative stress inhibits insulin-mediated glucose uptake is in agreement with some [10, 21, 26] but not all [6, 8, 19, 30] previous investigations. Discrepancies in the results of various studies can, in part, be explained by the differences in the experimental design, such as *in vivo* versus *in vitro* models and dose and duration of exposure to the compounds under investigation. Various levels of oxidative stress have been induced between 25 and 100 mU/ml GO for periods varying between 5 min and 24 h. In agreement with the results of previous studies [10, 26], we found that the induction of $H_2O_2$ via 100 mU/ml GO for 1 h in L6 myotubes impaired insulin-stimulated glucose transport. Importantly, however, the lack of effect of GO on selected proteins in the insulin signaling cascade suggests oxidative stress impairs cellular insulin-stimulated glucose transport via mechanism/s that are at least partly unrelated to these enzymes, at least under the current experimental conditions. Thus the precise mechanism by which ROS impairs glucose uptake remains to be determined. Nevertheless improved glucose uptake and insulin signaling by pretreatment with GT suggests that this antioxidant plays some role in counteracting effect of oxidative stress, and indeed potentially enhancing insulin signaling beyond insulin alone in certain circumstances.

The disconnect between glucose transport and insulin signaling (i.e. Akt and AS160 phosphorylation) in the current study strongly suggest that there are other mechanisms by which GT improved glucose

transport. Given that the pre-treatment with GT was more prolonged than the GO exposure, we speculate that reduced lipid peroxidation affords some improvement in cell membrane dynamics and interactions with transporters such as GLUT4, or some other local factor in the vicinity. The role of free radical attack in diabetes and in the cardiovascular complications of the disease has been documented largely through the effects of free radicals on lipids and proteins [9, 23, 24]. GT has been shown to act on the cell surface by preventing oxidation of LDL in cell membranes [15]. However, there is no evidence to suggest such mechanisms exist to prevent ROS-induced insulin resistance, and this is an area that requires further study.

The mechanism(s) by which $H_2O_2$ and other mediators of oxidative stress cause insulin resistance are largely unknown. A possible explanation for the inhibitory effect of $H_2O_2$ on insulin action is that it triggers an alteration in cellular redox balance due to prolonged exposure. Stress inducers, including $H_2O_2$, can function as signaling molecules to activate a number of stress sensitive serine/threonine kinase pathways linked to insulin resistance [5]. Insulin signaling enzymes offer a number of potential substrates for these activated serine kinases including the insulin receptor and the family of insulin receptor substrate proteins, which act to increase glucose transport through activating downstream enzymes such as Akt and AS160. Numerous agents that induce insulin resistance, such as TNF-$\alpha$, platelet-derived growth factor, angiotensin and hyperinsulinemia, all impair insulin signaling through induction of ROS [22, 29], which might explain their insulin de-sensitizing effects. In contrast, since we find that neither Akt nor AS160 phosphorylation were significantly impacted upon by GO treatment, the source of the ROS-induced impairment of insulin sensitivity lies, at least in part, elsewhere. Additional research that links the production of ROS and its removal by GT and other antioxidants, to impairment of insulin signaling events is clearly warranted to clarify this issue.

In conclusion, we found that acute oxidative stress impaired insulin-stimulated glucose transport in L6 myotubes, while pre-treatment with GT reversed this impairment. However, the disconnect between glucose uptake and the phosphorylation state of selected proteins in the insulin signaling cascade suggests that GT improves glucose transport via both insulin-dependent and independent mechanisms. Our results suggest that dietary supplementation with GT may play a preventive role in counteracting effect of oxidative stress on glucose uptake by skeletal muscle cells. In addition, identification of the molecular basis for this observation might lead to the discovery of pharmacological targets for novel therapies to prevent, reverse or delay the onset of insulin resistance and resultant pathogenesis.

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