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Gingerenone A Sensitizes the Insulin Receptor and Increases Glucose Uptake by Inhibiting the Activity of p70 S6 Kinase

Gingerenone → p70S6 (S6K1) ⊣ insulin sensitivity

*Junhong Chen, Jing Sun, Richard A. Prinz, Yi Li, and Xiulong Xu**

Scope: The bioactive constituents in ginger extract are responsible for anti-hyperglycemic effects and the underlying mechanisms are incompletely understood. Gingerenone A (Gin A) has been identified as an inhibitor of p70 S6 (S6K1), a kinase that plays a critical role in the pathogenesis of insulin resistance. This study aims to evaluate if Gin A can sensitize the insulin receptor by inhibiting S6K1 activity.

Methods and results: Western blot analysis reveals that Gin A induces phosphatidylinositide-3 kinase (PI3K) feedback activation in murine 3T3-L1 adipocytes and rat L6 myotubes, as evidenced by increased AKT^{S473} and $S6K1^{T389}$ but decreases $S6^{S235/236}$ and insulin receptor substrate 1 (IRS-1)^{S1101} phosphorylation. Western blot and immunoprecipitation analysis reveal that Gin A increases insulin receptor tyrosine phosphorylation in L6 myotubes and IRS-1 binding to the PI3K in 3T3-L1 adipocytes. Confocal microscopy reveals that Gin A enhances insulin-induced translocation of glucose transporter 4 (GLUT4) into the cell membrane in L6 cells. 2-NBDG (2-N-(Nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose) Fluorescent assay reveals that Gin A enhances insulin-stimulated glucose uptake in 3T3-L1 adipocytes and L6 myotubes.

Conclusions: Gin A overcomes insulin resistance and increases glucose uptake by inhibiting S6K1 activity. Gin A or other plant-derived S6K1 inhibitors could be developed as novel antidiabetic agents.

1. Introduction

More than one-third of American adults have metabolic syndrome, a condition characterized by the presence of insulin resistance, obesity, dyslipidemia, and hypertension.[1,2] Insulin resistance is the primary force driving the development of type 2 diabetes, a chronic disease with an unprecedented prevalence. Currently, more than a dozen antidiabetic drugs are available. However, the declining therapeutic efficacy or intolerable side effects after long-term patient use have often prevented the effective control of hyperglycemia.[3] Patients with inadequate or poor control of hyperglycemia often develop diabetic complications such as blindness, frequent limb infections, diabetic nephropathy, and cardiovascular disease. These complications are responsible for most diabetes-related morbidity and mortality.[3] There has been increasing interest in screening chemical compounds from the herb and plant extracts to develop safer and more effective antidiabetic drugs.

Insulin activates insulin receptor signaling by first phosphorylating the receptor itself. Activated insulin receptor in return phosphorylates other intracellular adaptor proteins such as insulin receptor substrates (IRS).[4,5] IRS interact with the p85 subunit of PI3K and activates PI3K, a kinase that phosphorylates and activates protein kinase B (AKT) (**Figure 1**A).[4,5] AKT regulates glucose

Dr. J. Chen, Dr. J. Sun
Institute of Comparative Medicine
Yangzhou 225009, Jiangsu Province, China
E-mail: xxl@yzu.edu.cn; xxu@rush.edu
Dr. J. Chen, Dr. J. Sun, Dr. X. Xu
College of Veterinary Medicine
Yangzhou 225009, Jiangsu Province, China
Dr. R. A. Prinz
Department of Surgery
NorthShore University Health System
Evanston, IL, USA
Dr. Y. Li
Lester and Sue Smith Breast Center
Baylor College of Medicine
Houston, TX 77030, USA
Dr. X. Xu
Jiangsu Co-innovation Center for Prevention and Control of Important Animal Infectious Diseases and Zoonosis
Yangzhou University
Yangzhou 225009, Jiangsu Province, China
Dr. X. Xu
Department of Cell and Molecular Medicine
Rush University Medical Center
Chicago, IL 60612, USA

The ORCID identification number(s) for the author(s) of this article can be found under https://doi.org/10.1002/mnfr.201800709

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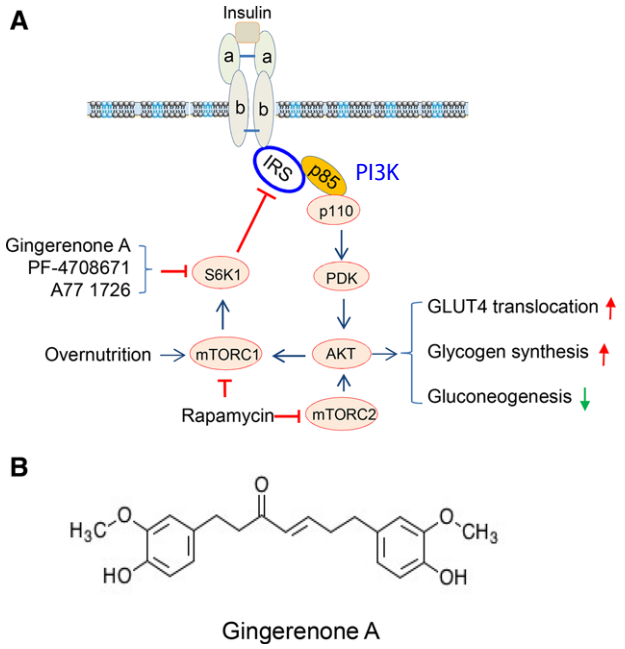
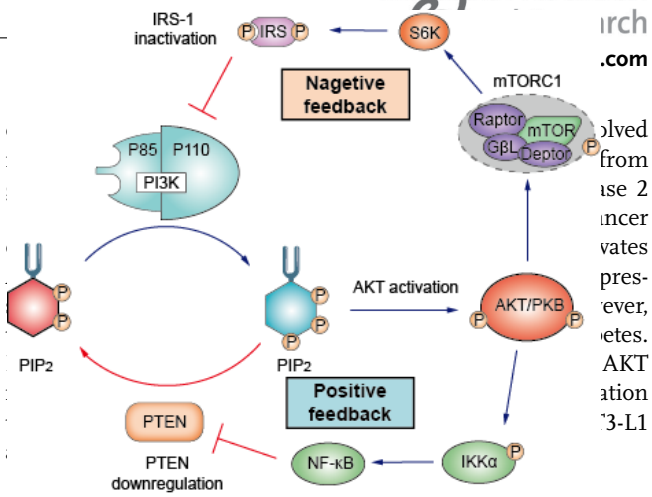


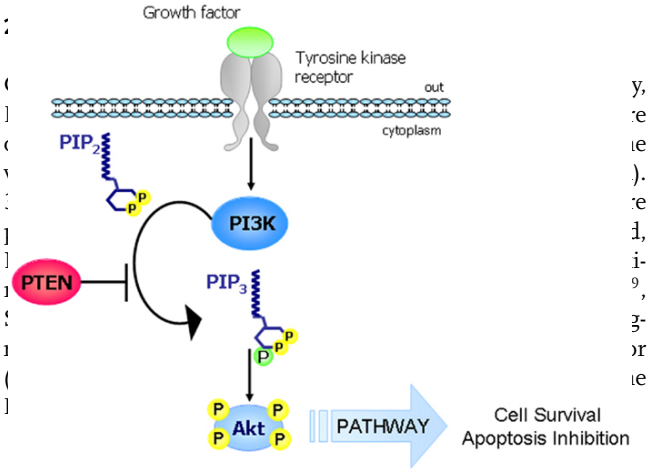
Figure 1. The mechanisms of S6K1 inhibitors-induced feedback activation of the PI3K pathway and insulin receptor sensitization. A) Mode of action of Gin A and two other S6K1 inhibitors, PF-4708671 and A77 1726. Overnutrition with high concentrations of fatty acids and amino acids leads to constitutive S6K1 activation, which phosphorylates IRS-1^{S1101}, leading to poor AKT activation. S6K1 inhibitors such as Gin A inhibit S6K1 activity and subsequently inhibit IRS-1^{S1101} phosphorylation, resulting in better interaction with the p85 subunit of PI3K and AKT activation. Activated AKT stimulates GLUT4 membrane translocation and glycogen synthesis but decreases gluconeogenesis. Chronic use of rapamycin leads to inhibition of both mTORC1 and mTORC2, thus exacerbating hyperglycemia. B) Chemical structure of Gin A.

metabolism by multiple mechanisms.[6,7] It inhibits gluconeogenesis, stimulates glycogen synthesis, and enhances glucose uptake in the insulin-sensitive tissues by triggering glucose transporter type 4 (GLUT4) translocation to the cell membrane (Figure 1A).[4,5] The mechanistic target of rapamycin (mTOR), a kinase downstream of AKT, is constitutively activated by nutrient overload, subsequently leading to hyperactivation of p70 S6 kinase (S6K1) (Figure 1A). S6K1 phosphorylates IRS-1 at serine 1101[7,8] and disrupts its interaction with PI3K, leading to poor AKT activation upon insulin stimulation.[9] S6K1 deficiency in S6K1$^{-/-}$ mice fed a high-fat diet (HFD) ameliorates obesity and hyperglycemia.[10] S6K1 is a key kinase that drives insulin resistance and induces obesity under nutrient overload conditions.[6]

While S6K1 appears to be a novel therapeutic target for the control of hyperglycemia, whether plant-derived S6K1 inhibitors can sensitize the insulin receptor, improve glucose metabolism, and control hyperglycemia has not been studied. Zingiber species belong to the Zingiberaceae (ginger) family and have been widely used as spice additives and plant medicines.[11] Ginger extracts and its pungent phenolic components can increase glucose uptake and reduce blood glucose level.[12–15] Gingerols and shogaols in ginger extract have been identified as two types of the bioactive ingredients mainly responsible for their antihyperglycemia and anti-obesity effects.[16–18] These compounds regulate glu-



2. Experimental Section



2.2. Cell Lines and Differentiation

L6 cells (a rat myoblast cell line) were grown in complete DMEM containing 10% fetal bovine serum. L6 myoblasts were differentiated into myotubes in DMEM by switching with the media containing 2% calf serum for 2 weeks. 3T3-L1 adipocytes (<15 passages) were differentiated as described by Zebisch et al.[21] An adipocyte-like phenotypic change in >95% of the cells was considered to successfully induce cell differentiation. Both L6 and 3T3-L1 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA).

2.3. Western Blot

3T3-L1 adipocytes were cultured in serum-free medium overnight, whereas L6 myotubes were cultured in serum-free medium for 2 h. Gin A at indicated concentrations (2.5–40 μM) or PF-4708671 (10 μM) was added and incubated for the indicated time, followed by stimulation with 100 nM insulin for the indicated time. Unstimulated cells were included as a negative control. Cells were harvested and lysed in NP-40 lysis buffer containing 1% NP-40; 50 mM Tris-HCl, pH 8.0; 150 mM NaCl; 5 mM EDTA; 10 μg mL^{-1} aprotinin; 10 μg mL^{-1} leupeptin; 1 mM phenylmethylsulfonyl fluoride; and 2 mM sodium pervanadate. Protein phosphorylation and total amounts of proteins were analyzed by Western blot with the indicated antibodies. NIH

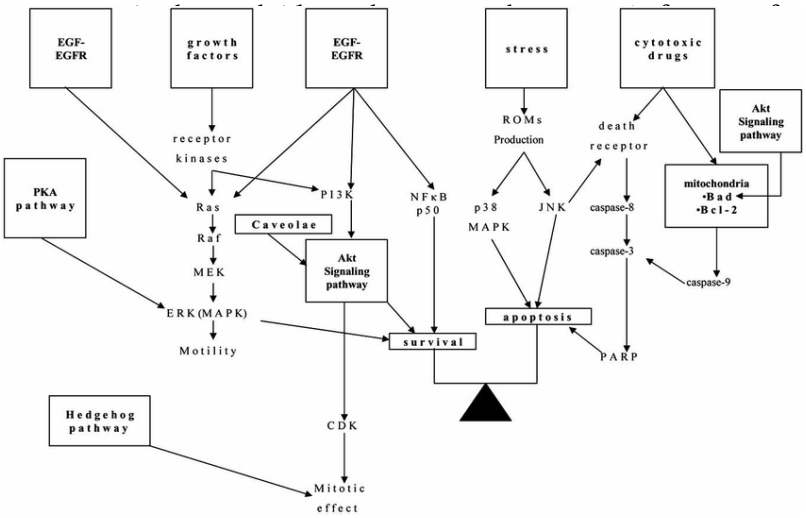
Image-J software was used to quantify the density of the bands followed by normalization with the arbitrary units of their corresponding total proteins. Results were presented as the mean ± SD from three independent experiments in bar graphs.

2.4. IRS-1 Binding to the PI-3 Kinase

3T3-L1 adipocytes were grown in serum-free medium overnight and then incubated either in essential balanced salt solution (EBSS) or EBSS containing 2× amino acids (AA) in the absence or presence of Gin A or PF-4708671 (20 μM) for 4 h. Cells were stimulated with insulin (100 nM) for 10 min. Cells were harvested and lysed in NP-40 buffer, followed by immunoprecipitation with an anti-p85 rabbit monoclonal antibody. Immunoprecipiates were analyzed by electrophoresis, followed by Western blot with anti-p85 and anti-IRS-1 antibodies.

2.5. Visualization of GLUT4 Membrane Translocation

L6 cells were seeded on coverslips and then transiently transfected with mCherry-Glut4-myc expression vector DNA using TurboFect Reagent according to the manufacturer's instruction. After incubation for 24 h, the cells were treated with Gin A 10 μM for 4 h in EBSS containing 10% FBS with or without 2× AA. Cells were left unstimulated or stimulated with 100 nM insulin for 30 min. The coverslips were fixed in 4% paraformaldehyde at room temperature for 10 min. The coverslips were mounted with 50% glycerin in PBS containing 4,6-diamidino-2-phenylindole (0.5 μg mL^{-1}; Sigma Chemical Co.). mCherry-tagged Glut4 fluorescence was visualized under a Leica LP8 confocal microscope. The numbers of cells with GLUT4 membrane staining in ten randomly selected fields from each treatment were divided by the total numbers of mCherry-GLUT4-positive cells. Percents of the GLUT4 membrane-positive cells were calculated and presented



2.7. Dosage Information

L6 and 3T3-L1 cells were cultured in the absence or presence of Gin A at concentrations of 10–40 μM for 4 h. No visible cytotoxicity such as cell floating, rounding, or ruffling etc. was observed. Gin A causes cytotoxicity only in cancer cells that are addicted to an oncogene and does not have cytotoxicity in untransformed cells such as L6 and 3T3-L1 cells[19,20]. Suk et al.[20] reported that Gin A at 40 μM does not cause 3T3-L1 adipocytes cytotoxicity after incubation for 6 days. Likewise, there was no cytotoxicity in HEK293 cells after incubation with Gin A (50 μM) for 24 or 48 h (X. Xu, unpublished observations).

2.8. Statistical Analysis

An unpaired Student's *t*-test was used to evaluate if there were significant differences in glucose uptake in 3T3-L1 adipocytes and L6 myotubes in various treatment groups. A p-value <0.05 was considered statistically significant. All statistics analyses were conducted using a GraphPad Prism 6 software.

3. Results

3.1. Gin A Induces PI3K Feedback Activation Under Insulin-Sensitive Conditions

A prior study has shown that Gin A inhibits the activity of S6K1 and induces AKT feedback activation in the HCT116 colorectal cancer cell line.[19] We first evaluated the ability of Gin A to inhibit S6K1 activity and to induce the feedback activation of the PI3K pathway in 3T3-L1 adipocytes and in L6 myotubes. As shown in **Figure 2**A, insulin induced AKT^{S473}, S6K1^{T389}, ERK$^{T202/204}$, and S6$^{S235/236}$ phosphorylation in 3T3-L1 adipocytes. Gin A enhanced insulin-induced phosphorylation of AKT^{S473} at 10, 20, and 40 μM in a dose-dependent manner. Interestingly, S6K1^{T389} phosphorylation was significantly increased by Gin A at 2.5 and 5 μM but started to decline at 10, 20, and 40 μM. Consistently, Gin A enhanced insulin-induced AKT^{S473} phosphorylation in L6 myotubes in a dose-independent manner (Figure 2B). S6K1^{T389} phosphorylation levels were lower in L6 myotubes treated with Gin A at high concentrations (20 and 40 μM) than that at low concentrations (2.5–10 μM). Gin A increased ERK$^{T202/204}$ phosphorylation but decreased S6$^{S235/236}$ phosphorylation in both 3T3-L1 adipocytes and L6 myotubes in a dose-dependent manner. PF-4708671 was included as a positive control. It significantly increased AKT^{S473}, S6K1^{T389}, and ERK$^{T202/204}$ phosphorylation but decreased S6$^{S235/236}$ phosphorylation in both 3T3-L1 adipocytes and L6 myotubes (Figure 2). The observations of increased S6K1^{T389} phosphorylation by Gin A and PF-4708671 is consistent with numerous studies showing that inhibition of S6K1 activity leads to increased S6K1 phosphorylation.[22,23]

3.2. Gin A Induces PI3K Feedback Activation in 3T3-L1 Adipocytes Under Insulin-Resistant Conditions

We next determined the effect of Gin A on insulin signaling in these two cell lines cultured in the presence of 2× amino acids (AA), a condition of insulin resistance. As shown in **Figure 3**A, Gin A at 10 μM enhanced insulin-induced S6K1^{T389}

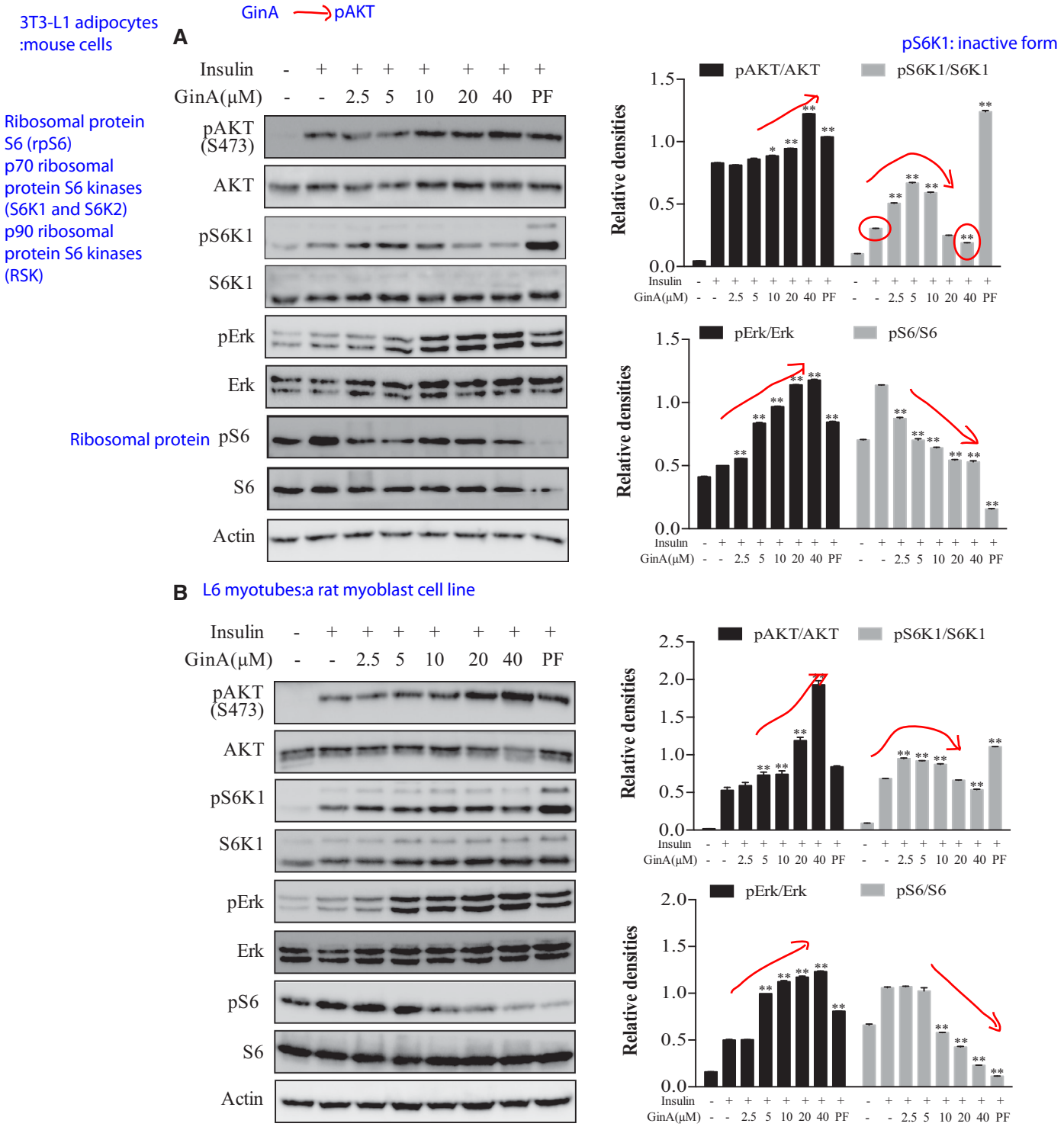


Figure 2. Gin A induces feedback activation of the PI3K and MAPK pathways. A) 3T3-L1 adipocytes were starved in serum-free media overnight and then treated with Gin A or PF-4708671 (10 μM) for 5 h. The cells were left unstimulated or stimulated with insulin (100 nM) for 10 min. B) L6 myotubes were starved in serum-free media for 2 h and then treated with Gin A or PF-4708671 (10 μM) for 2 h. The cells were left unstimulated or stimulated with insulin (100 nM) for 5 min. Cells were harvested and analyzed for the phosphorylation of $S6K1^{T389}$, AKT^{S473}, $ERK^{T202/204}$, and $S6^{S235/236}$, followed by reprobing with their specific antibodies for total protein. β-Actin was included as a loading control. Relative protein phosphorylation was determined by analyzing the density of the bands and presented as bar graphs. The results are the mean ± SD from three experiments. $^{*}p < 0.05$; $^{**}p < 0.01$, compared to insulin-stimulated control.

Branched-chain and aromatic amino acids were strongly associated with insulin resistance

EBSS: essential balanced salt solution

2x aa is cause or effect of insulin resistance?

mTORC1 --> S6K --> S6

3T3-L1 adipocytes

insulin resistance: 2× amino acids (AA)

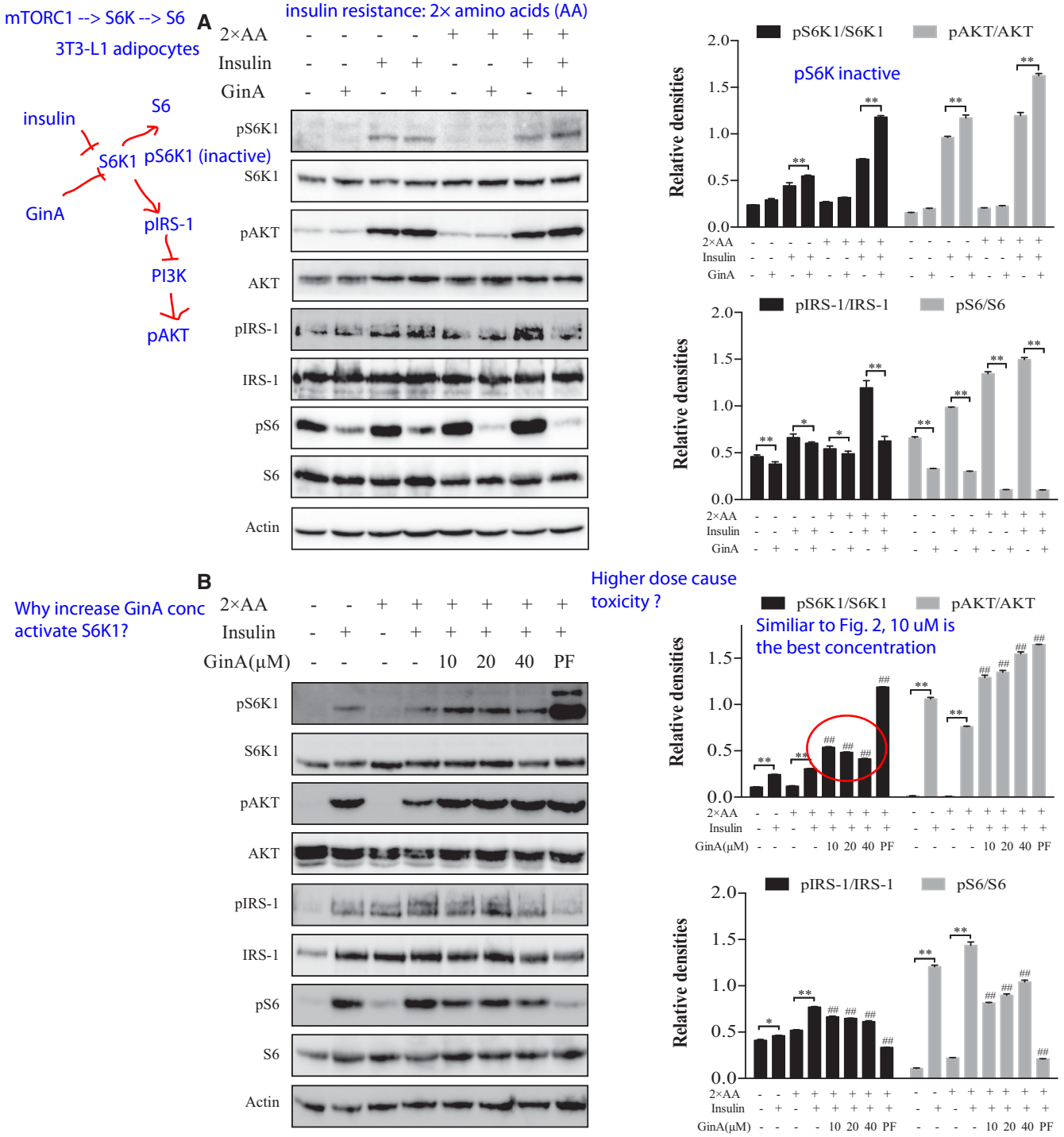


Figure 3. Gin A induces feedback activation of the PI3K pathway in 3T3-L1 adipocytes under insulin resistance conditions. 3T3-L1 adipocytes were starved in serum-free medium overnight and incubated in EBSS or EBSS containing 2× AA, then treated with or without Gin A (10 μM; A) or with the indicated concentrations of Gin A or PF-4708671 (20 μM; B) for 5 h. The cells were left unstimulated or stimulated with insulin (100 nM) for 10 min. Cells were harvested and analyzed for the phosphorylation of S6K1^{T389}, AKT^{S473}, S6$^{S235/236}$, and IRS-1^{S1101}, followed by reprobing with their specific antibodies for total protein. β-Actin was included as a loading control. Relative protein phosphorylation was determined by analyzing the density of the bands and presented as bar graphs. The results are the mean ± SD from three experiments. $^{*}p < 0.05$; $^{**}p < 0.01$, $^{\#}p < 0.05$; $^{\#\#}p < 0.01$, compared to the control (with insulin and 2× AA but without drug).

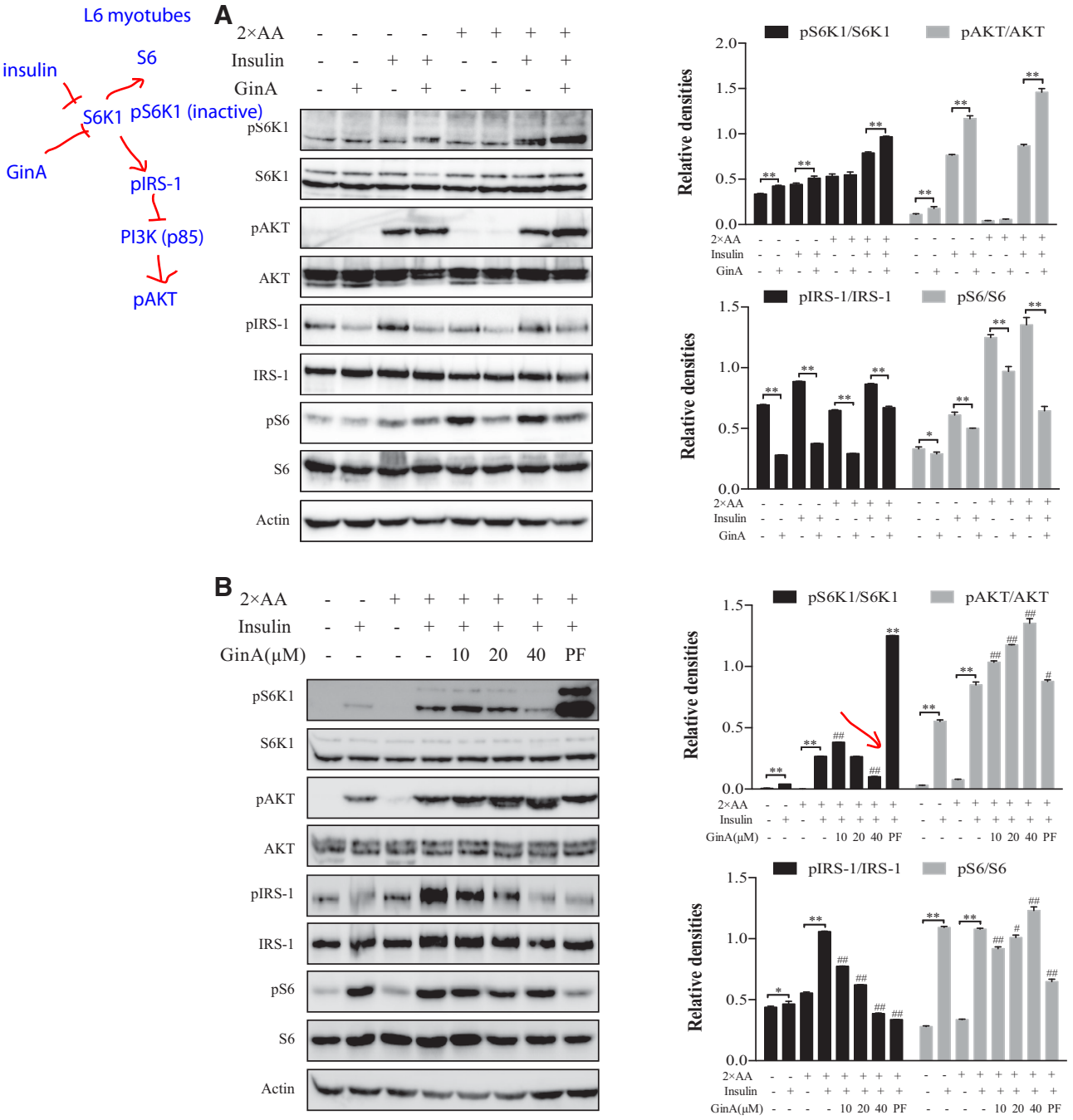


Figure 4. Gin A induces feedback activation of the PI3K pathway in L6 myotubes under insulin resistance conditions. L6 myotubes were starved in serum-free media for 2 h and incubated with EBSS or EBSS containing 2× AA, then treated with or without Gin A (10 μM; A) or treated with the indicated concentrations of Gin A or PF-4708671 (20 μM; B) for 2 h. The cells were left unstimulated or stimulated with insulin (100 nM) for 5 min. Cells were harvested and analyzed for the phosphorylation of S6K1^{T389}, S6$^{S235/236}$, IRS-1^{S1101}, and AKT^{S473}, followed by reprobing with their specific antibodies for total protein. β-Actin was included as a loading control. Relative protein phosphorylation was determined by analyzing the density of bands and presented as bar graphs. The results are the mean ± SD from three experiments. $^{*}p < 0.05$; $^{**}p < 0.01$, $^{\#}p < 0.05$; $^{\#\#}p < 0.01$, compared to the control (with insulin and 2× AA but without drug).

and AKT^{S473} phosphorylation more effectively in the presence of 2× AA than in its absence. Likewise, Gin A more effectively inhibited S6$^{S235/236}$ and IRS-1^{S1101} phosphorylation in the presence of 2× AA than in its absence. Dose response revealed that Gin A at concentrations of 20 and 40 μM was less effective in inducing S6K1 phosphorylation than that with Gin A at 10 μM (Figure 3B). In contrast, Gin A increased AKT^{S473} but decreased IRS-1^{S1101} phosphorylation in a dose-dependent manner. Gin

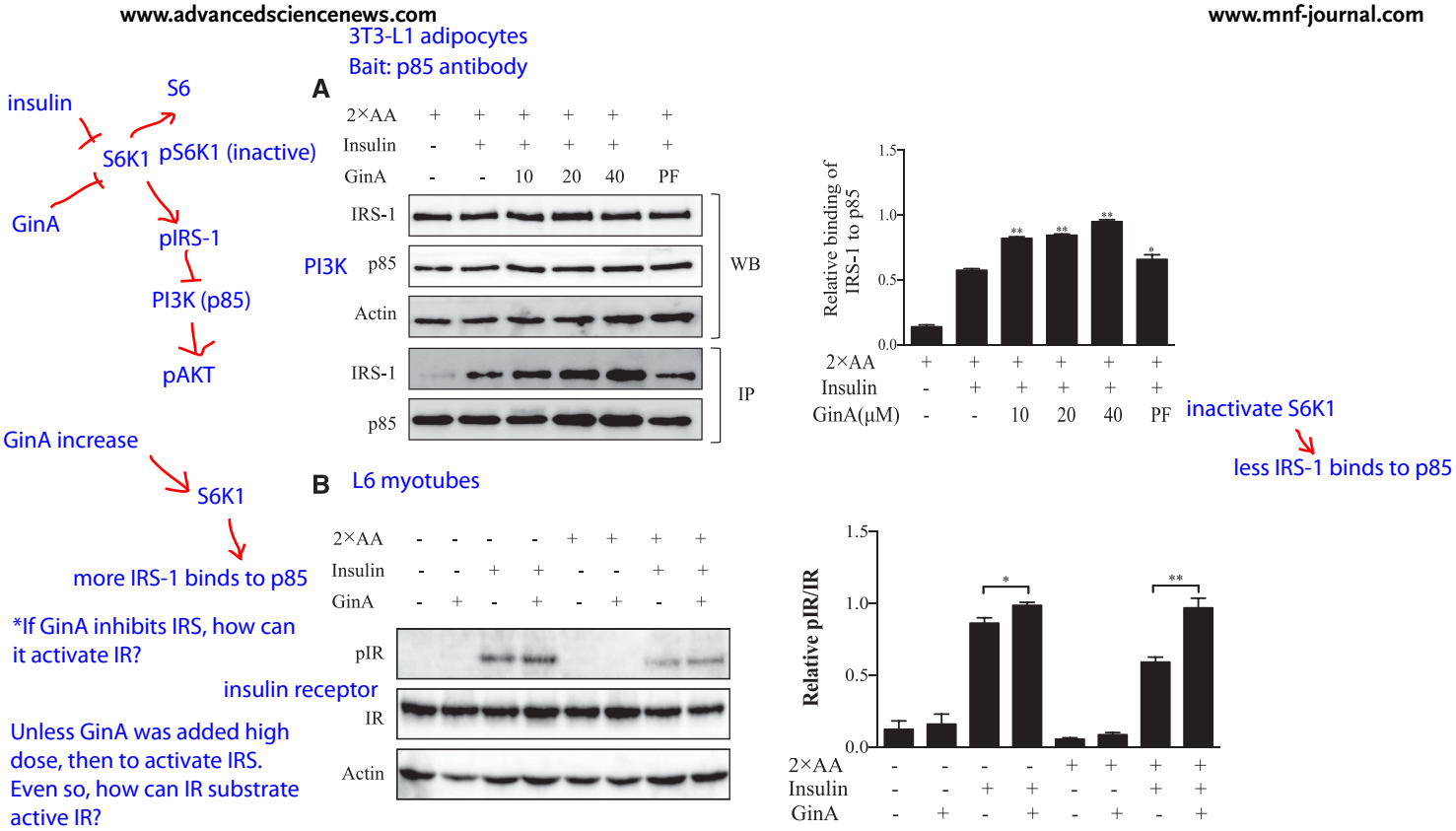


Figure 5. GinA sensitizes insulin receptor signaling. A) 3T3-L1 adipocytes were starved in serum-free media overnight and incubated with EBSS or EBSS containing 2× AA, then treated with the indicated concentrations of Gin A or PF-4708671 (20 μM) for 4 h. The cells were left unstimulated or stimulated with insulin (100 nM) for 10 min. Cells lysates were immunoprecipitated with an anti-p85 antibody followed by probing with anti-p85 and anti-IRS-1 antibodies. B) 3T3-L1 adipocytes were treated as (A). Cell lysates were analyzed for IR^{Y1146} phosphorylation, followed by reprobing with an antibody against total IR protein. β-Actin was included as a loading control. Relative IRS-1 binding to the p85 subunit of PI3K (A) or relative IR phosphorylation levels (B) were determined by analyzing the density of the bands and presented as bar graphs. The results are the mean ± SD from three experiments. $^{*}p < 0.05$; $^{**}p < 0.01$, compared to the control (with insulin and 2× AA but without drug).

A did decrease $S6^{S235/236}$ phosphorylation but not in a dose-dependent manner. PF-4708671 was included as a positive control. Again, it increased AKT^{S473} and $S6K1^{T389}$ but decreased $S6^{S235/236}$ and $IRS\text{-}1^{S1101}$ very effectively in 3T3-L1 adipocytes in the presence of 2× AA (Figure 3B).

3.3. Gin A Induces PI3K Feedback Activation in L6 Myotubes Under Insulin-Resistant Conditions

We also assessed the effect of Gin A on insulin signaling in L6 myotubes cultured in the presence of 2× AA. As shown in **Figure 4**A, Gin A at 10 μM enhanced insulin-induced $S6K1^{T389}$ and AKT^{S473} phosphorylation more effectively in the presence of 2× AA than that in its absence. Gin A inhibited $IRS\text{-}1^{S1101}$ and $S6^{S235/236}$ phosphorylation in the absence or presence of 2× AA. Dose response analysis revealed that Gin A at 10 μM maximally increased S6K1 phosphorylation (Figure 4B). Gin A increased AKT^{S473} but decreased $IRS\text{-}1^{S1101}$ phosphorylation in a dose-dependent manner. Gin A decreased $S6^{S235/236}$ phosphorylation but not in a dose-dependent manner. PF-4708671 was included as a positive control. Again, it increased AKT^{S473} and $S6K1^{T389}$ but decreased $S6^{S235/236}$ and $IRS\text{-}1^{S1101}$ very effectively in L6 myotubes in the presence of 2× AA (Figure 4B).

3.4. Gin A Increases the Interaction of the PI3K with IRS-1 and Sensitizes the Insulin Receptor

We next determined whether AKT feedback activation resulted from increased interaction between IRS-1 and the PI3K. As shown in **Figure 5**A, insulin increased the binding of the p85 subunit of the PI3K to IRS-1 in 3T3-L1 adipocytes. Gin A further increased the interaction of IRS-1 with the PI3K in a dose-dependent manner. PF-4708671 included as a positive control also increased the binding of p85 subunit of the PI3K to IRS-1 (Figure 5A). Total IRS-1 and p85 levels were not changed in 3T3-L1 adipocytes incubated in the absence or presence of Gin A for 4 h. Gin A increased insulin-stimulated tyrosine phosphorylation of the insulin receptor slightly more effectively in L6 myotubes in the presence of 2× AA than that in its absence (Figure 5B).

3.5. Gin A Increases GLUT4 Translocation to Cell Membrane

AKT activation triggers the translocation of GLUT4 from cytoplasmic vesicles to the cell membrane.[7,24] Here, we tested if AKT feedback activation by Gin A led to GLUT4 translocation from the cytoplasm to the cell membrane. As shown in **Figure 6**A, GLUT4 was present mostly in the cytoplasm of DMSO-treated L6 cells but

was translocated to the membrane of L6 cells upon insulin stimulation (Figure 6A). Gin A slightly increased GLUT4 translocation from the cytoplasm into the cell membrane in the absence of 2× AA. In contrast, Gin A significantly enhanced insulin-stimulated GLUT4 translocation from the cytoplasm to the cell membrane in the presence of 2× AA (Figure 6A, B).

3.6. Gin A Enhances Insulin-Stimulated Glucose Uptake

Having shown that Gin A increased GLUT4 membrane translocation, we next determined the ability of Gin A to increase glucose uptake. As shown in **Figure 7**A, intracellular glucose levels were significantly increased by insulin stimulation in 3T3-L1 adipocytes and L6 myotubes in the absence of 2× AA. The ability of insulin to stimulate glucose uptake was slightly weakened in 3T3-L1 adipocytes and L6 myotubes by 2× AA. Gin A further increased intracellular glucose levels in insulin-stimulated 3T3-L1 adipocytes in a dose-dependent manner (Figure 7A) and in L6 myotubes (Figure 7B). PF-4708671 was included as a positive control. It also increased intracellular glucose levels in 3T3-L1 adipocytes (Figure 7A) and L6 myotubes (Figure 7B). These results collectively suggest that elevated GLUT4 membrane translocation by Gin A due to insulin receptor sensitization is responsible for increased glucose uptake.

4. Discussion

It has been long recognized that ginger and ginger extract have beneficial effects on glucose and lipid metabolism.[25] Most studies have focused on the antidiabetic and anti-obesity effects of gingerols and shogaols extracted from ginger.[25–28] These compounds exert their biological activities largely by activating AMPK, an energy sensor that regulates glucose and lipid metabolism.[25–28] Though S6K1 plays a central role in the metabolic syndrome and type 2 diabetes, whether targeting S6K1 with natural products can improve glucose metabolism remains unexplored. In the present study, we report that Gin A, a constituent in ginger extract and an inhibitor of S6K1, was able to sensitize the insulin receptor, to increase GLUT4 translocation into the plasma membrane, and to stimulate glucose uptake in adipocytes and myotubes. Our study is the first to report that a plant-derived S6K1 inhibitor can improve glucose metabolism by directly targeting a crucial enzyme involved in insulin resistance. Since the relative amount of Gin A and other bioactive agents such as gingerols and shogaols in ginger is unknown, how significantly Gin A contributes to the antihyperglycemic effect of Gin A is not clear, exploring S6K1 inhibitors from natural products could be a vital approach to discovering novel antidiabetic drugs or dietary supplements for the control of hyperglycemia.

S6K1 has been recently pursued as a molecular target for sensitizing the insulin receptor and for controlling hyperglycemia.[29] Shum et al.[29] reported that PF-4708671, a specific inhibitor of S6K1, sensitizes the insulin receptor and enhances glucose uptake. We recently identified S6K1 as a molecular target of A77 1726, the active metabolite of anti-rheumatoid arthritis (RA) drug leflunomide.[30] A77 1726 induces feedback activation of the PI3K pathway in A375 melanoma cells,[31] NSC34 motoneuron cells,[32] 3T3-L1 adipocytes, and L6 myotubes.[33] Byun et al.[19] reported that Gin A inhibits the activity of S6K1, leading to increased AKT phosphorylation in the HCT116 colorectal cancer cell line. Consistent with these observations, our present study showed

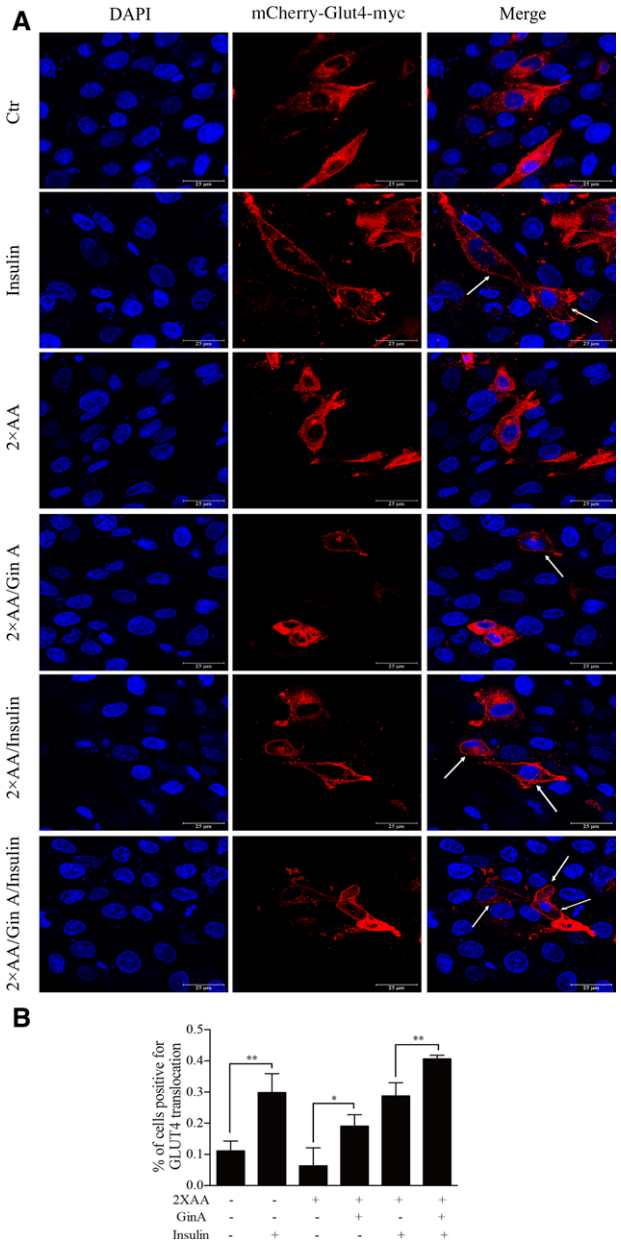


Figure 6. Gin A enhances insulin-stimulated GLUT4 translocation to the cell membrane. A) L6 cells transfected with mCherry-Glut4-myc were treated with Gin A (10 μM) for 4 h in EBSS containing 10% FBS with or without 2× AA. Cells were left unstimulated or stimulated with insulin (100 nM) for 30 min. After fixation, mCherry-tagged GLUT4 fluorescence was visualized under a Leica LP8 confocal microscope. Arrows denote the mCherry-tagged GLUT4 translocation to the cell membrane. B) Quantification of GLUT4 translocation to the plasma membrane. The data represent the mean ± SD from one of three experiments with similar results. $^{*}p < 0.05$; $^{**}p < 0.01$.

At such conc of GinA, S6K1 is no longer inhibited.

2-NBDG is a fluorescent tracer used for monitoring glucose uptake into living cells

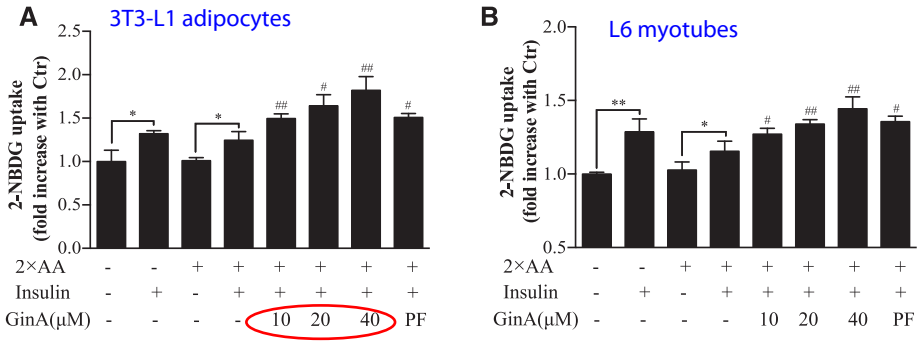


Figure 7. Gin A increases glucose uptake. 3T3-L1 adipocytes A) and L6 myotubes B) were starved in serum- and glucose-free media overnight and incubated with the indicated concentrations of Gin A or PF-4708671 in EBSS or EBSS containing 2× AA for 1 h. 2-NBDG (50 μM) was added for another 1 h. The cells were left unstimulated or stimulated with insulin (50 nM) for 30 min. The cultured media were removed, followed by rinsing three times with PBS. The fluorescence intensity was read in a plate reader with the excitation wavelength of 485 nm and an emission wavelength of 535 nm. Data represents the mean ± SD of one experiment in triplicate. The experiments were repeated three times with similar results. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{\#}p < 0.05$; $^{\#\#}p < 0.01$, compared to the control (with insulin and 2× AA but without drug).

that Gin A inhibited $S6^{S235/236}$ and $IRS\text{-}1^{S1101}$ phosphorylation and induced feedback activation of the PI3K pathway in L6 myotubes and 3T3-L1 adipocytes under normal and insulin resistance conditions. Gin A enhanced insulin-induced insulin receptor tyrosine phosphorylation and increased IRS-1 binding to PI3K. In the presence of high amino acid concentrations, Gin A enhanced insulin-induced GLUT4 translocation to the plasma membrane in L6 cells and increased glucose uptake in 3T3-L1 adipocytes and L6 myotubes. These observations suggest that Gin A improves insulin receptor signaling by inhibiting $IRS\text{-}1^{S1101}$ phosphorylation.

We noticed that Gin A did not increase S6K1 phosphorylation in a dose-dependent manner. S6K1 phosphorylation levels in 3T3-L1 adipocytes and L6 myotubes treated with Gin A at 20–40 μM were lower than when it was used at 10 μM (Figures 2–4). Suk et al.[20] recently reported that Gin A at 40 μM induced AMP-activated protein K (AMPK) phosphorylation in 3T3-L1 adipocytes and in epidermal white adipose tissue in vivo. Since AMPK suppresses mTORC1 activity directly by phosphorylating Raptor and indirectly by phosphorylating TSC2,[34,35] we speculate that AMPK activation by Gin A at high concentrations leads to the suppression of mTOR activity, thus restraining the maximal increase of S6K1 phosphorylation. However, declining S6K1 phosphorylation did not lead to better inhibition of S6 phosphorylation (Figures 3B and 4B). Instead, S6 phosphorylation levels in 3T3-L1 adipocytes and L6 myotubes under insulin-resistant conditions treated with high concentrations of Gin A (20 and 40 μM) were not significantly lower than that treated with Gin A at 10 μM. We speculate that $p90^{RSK}$ activation by feedback-activated ERK phosphorylates S6,[36] thus preventing a dose-dependent inhibition of S6 phosphorylation (Figures 3B and 4B).

There has been growing interest in finding natural products from herbs and plants to control the metabolic syndrome.[37,38] Some phytochemicals in spice ingredients, herbal products, and dietary supplements can control hyperglycemia and obesity by improving glucose and lipid metabolism.[38–41] Ginger has been long considered a herbal medicine and has beneficial effects on a variety of ailments, including inflammation, infection, cancer, diabetes, obesity, and cardiovascular diseases.[18,26] Several bioactive constituents in ginger extract have been identified and characterized, including gingerols, shogoals, and paradols. Gingerols and shogoals largely exert their antihyperglycemic and anti-obesity effects by activating AMPK.[16–18] 6-Dihydroparadol increases cholesterol efflux in a human THP-1 macrophage cell line by inhibiting proteasomal activity and increasing the protein levels of ATP-binding cassette transporters, ABCA1 and ABCG1.[42] This bioactivity may contribute to the anti-atherogenic effect of ginger.[42] Suk et al. recently reported that Gin A activates AMPK in vitro in 3T3-L1 adipocytes and in vivo in epidermal white adipose tissue.[20] Consistent with these observations, we found that Gin A induced AMPK phosphorylation in both 3T3-L1 adipocytes and L6 myotubes (data not shown). How Gin A activates AMPK is not known. Our recent study showed that inhibition of S6K1 activity by A77 1726 and PF-4708671 activates AMPK through the TGF-β-activated kinase 1 (TAK1) in A375 melanoma cells and in NSC34 motoneuron cells.[31,32] In addition, Gin A may also activate AMPK by elevating the AMP levels since S6K1 deficiency leads to increased AMP/ATP ratios and AMPK activation in the skeletal muscle tissues and myotubes of S6K1-deficient mice.[43,44] Since S6K1 can bind and phosphorylate AMPK α2 at S491, leading to the inhibition of AMPK activity,[45] Gin A may also activate AMPK by inhibiting S6K1-mediated $AMPK^{S491}$ phosphorylation. Although we cannot rule out the possibility that Gin A may target other molecules to regulate glucose and lipid metabolism, Gin A may activate AMPK through multiple mechanisms, but all by inhibiting S6K1 activity. It remains to be determined if AMPK activation by other constituents such as gingerols or shogaols in ginger extract[16–18] is also mediated by inhibition of S6K1 activity.

S6K1 plays a critical role in the pathogenesis of type 2 diabetes.[6] We and others recently reported that S6K1 inhibitors such as PF-4708671 and leflunomide can control hyperglycemia in type 2 diabetic mouse models.[29,33] The antihyperglycemic and anti-obesity effects of leflunomide have been observed in leflunomide-treated RA patients.[46,47] Leflunomide could be a leading candidate for treating type 2 diabetes since the side effects of this clinically approved drug have been well characterized.[48,49] However, A77 1726 has other biochemical activities, including inhibition of protein tyrosine kinase activities and inhibition of pyrimidine nucleotide synthesis.[48] Leflunomide could be

particularly useful for treating RA patients with diabetes, but its immunosuppressive effect could be a concern in most other patients.[33,48] Food-derived S6K1 inhibitors or S6K1 inhibitor-enriched dietary supplements such as Gin A and ginger extract should have a relatively safe profile and could be developed as antidiabetic drugs.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

gingerenone A, glucose uptake, insulin receptors, insulin resistance, p70 S6 kinase

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