Ras Inhibition Induces Insulin Sensitivity and Glucose Uptake

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Abstract

Background: Reduced glucose uptake due to insulin resistance is a pivotal mechanism in the pathogenesis of type 2 diabetes. It is also associated with increased inflammation. Ras inhibition downregulates inflammation in various experimental models. The aim of this study was to examine the effect of Ras inhibition on insulin sensitivity and glucose uptake, as well as its influence on type 2 diabetes development.

Methods and Findings: The effect of Ras inhibition on glucose uptake was examined both in vitro and in vivo. Ras was inhibited in cells transfected with a dominant-negative form of Ras or by 5-fluoro-farnesylthiosalicylic acid (F-FTS), a small-molecule Ras inhibitor. The involvement of IkB and NF-κB in Ras-inhibited glucose uptake was investigated by immunoblotting. High fat (HF)-induced diabetic mice were treated with F-FTS to test the effect of Ras inhibition on induction of hyperglycemia. Each of the Ras-inhibitory modes resulted in increased glucose uptake, whether in insulin-resistant C2C12 myotubes in vitro or in HF-induced diabetic mice in vivo. Ras inhibition also caused increased IkB expression accompanied by decreased expression of NF-κB. In fat-induced diabetic mice treated daily with F-FTS, both the incidence of hyperglycemia and the levels of serum insulin were significantly decreased.

Conclusions: Inhibition of Ras apparently induces a state of heightened insulin sensitization both in vitro and in vivo. Ras inhibition should therefore be considered as an approach worth testing for the treatment of type 2 diabetes.

Introduction

Insulin resistance is defined as impaired sensitivity to insulin in its main target organs (muscle, liver and adipose tissues), and is considered a hallmark of type 2 diabetes [1]. Insulin levels regulate glucose uptake by a variety of mechanisms, including induction of glucose transporter 4 (Glut4) expression, enhancement of translocation of the transporter to the muscle tissue membranes, reduction of free fatty acid (FFA) secretion from adipocytes, and inhibition of gluconeogenesis in the liver. Resistance to insulin results in increased concentrations of circulating FFA, which inhibits glucose uptake by muscle cells and increases glucose production by the liver [2].

Recent findings point to interrelationships between inflammation, insulin resistance, and type 2 diabetes. Lipid accumulation in the adipose tissue and expansion of the fat mass can initiate an inflammatory process, accompanied by local production and secretion of pro-inflammatory cytokines and chemokines [3,4]. Pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α) reduce insulin sensitivity in muscle tissue and stimulate hepatic lipogenesis and hyperlipidemia [5,6,7]. Hepatic steatosis promotes low-grade inflammation via activation of nuclear factor-κB (NF-κB) [8]. The affected adipose, muscle and liver tissues together create an inflammatory milieu that promotes insulin resistance locally [9].

In an insulin-resistant state, serine kinases phosphorylate insulin receptor substrate (IRS), which results in inhibition of insulin signaling. A prominent participant in this process is the inhibitor of κB kinase (IKK), which phosphorylates, among other molecules, the insulin receptor. It also phosphorylates IkB, inducing the release of nuclear factor-κB (NF-κB) from IkB and allowing it to enter the nucleus [10]. NF-κB promotes upregulation of mediators that enhance inflammation and induce disease progression [11,12,13].

A prominent protein family that participates in the regulation of intracellular signal transduction and exerts a major impact on inflammation is the family of Ras GTPases [14,15]. These small (~21 kDa) proteins consist of molecular switches that regulate cell growth, differentiation, survival, migration and death [15,16,17,18,19,20,21,22]. Ras is crucially involved in the proper activity of many cell types, including immune cells. Therefore, its abnormal involvement in cancer and autoimmune diseases has been the subject of intensive research [23,24,25,26], with many studies aimed at understanding the possible involvement of Ras signaling in the disease and at developing selective inhibition of the active Ras protein. A well characterized protein activated by Ras is

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A. Insulin-resistant C2C12 myotubes were transfected with DN-Ras-GFP or GFP plasmid (pGFP) and fluorescent glucose uptake was measured by flow cytometry. Representative histograms of glucose uptake are presented ($n=4$). B. Statistical analysis of the results is presented as means \( \pm \) S.D. $^* P<0.05$. C. IκB, NF-κB and tubulin expression in the DN-Ras transfected or GFP-transfected myotubes were assayed by western blotting, as described in Material and Methods. Representative blots are presented ($n=4$). D. Densitometry of IκB and NF-κB expression. $^* P<0.05$ compared to control.

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Figure 1. Inhibition of Ras in vitro by DN-Ras increases glucose uptake and alters IκB/ NF-κB expression. A. Insulin-resistant C2C12 myotubes were transfected with DN-Ras-GFP or GFP plasmid (pGFP) and fluorescent glucose uptake was measured by flow cytometry. Representative histograms of glucose uptake are presented ($n=4$). B. Statistical analysis of the results is presented as means \( \pm \) S.D. $^* P<0.05$. C. IκB, NF-κB and tubulin expression in the DN-Ras transfected or GFP-transfected myotubes were assayed by western blotting, as described in Material and Methods. Representative blots are presented ($n=4$). D. Densitometry of IκB and NF-κB expression. $^* P<0.05$ compared to control.

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the AKT protein. Through stimulation of PI3K, Akt/PKB kinase is activated and phosphorylates the IKK, which in turn activates NF-kB. Inhibition of Ras can therefore attenuate NF-kB activation and reduce the inflammatory process [27].

S-trans-farnesylthiosalicylic acid (FTS, Salirasib) is a small synthetic molecule that acts as a potent Ras inhibitor by competing with the anchoring of active Ras to the plasma membrane. Our group has described a number of FTS analogs that also act as Ras inhibitors, the most potent being 5-fluoro-FTS (F-FTS) [28]. The effect of FTS and its analog was studied in various animal models of immune-mediated experimental disorders and found to significantly attenuate disease progression [29]. The attenuation was accompanied by altered gene expression in Ras signaling pathways, including the NF-kB signaling cascade [23,24,25,26,30,31,32].

In the present study we attempted to gain a better understanding of the effects of Ras inhibition on insulin resistance and type 2 diabetes by treating differentiated myotubes in vitro and high-fat (HF)-induced diabetic mice in vivo with DN-Ras or the synthetic Ras inhibitor F-FTS. We examined the effects of such treatment on glucose uptake and cellular signaling pathways, with particular focus on NF-kB-dependent signaling cascades. We found that treatment with the Ras inhibitor, F-FTS, reduced insulin resistance in vitro and attenuated type 2 diabetes in vivo. The effects of Ras inhibition were mediated by the IκB/NF-κB cascade.

Materials and Methods

Induction of insulin resistance in cell culture

Mouse C2C12 myoblasts (generously provided by Prof. David Yaffe) were maintained in DMEM supplemented with 10% fetal bovine serum (FBS), 50 U/ml penicillin, and 50 μg/ml streptomycin. When cells reached confluence, the medium was replaced by differentiation medium containing DMEM and 2% horse serum, which was changed every other day. After 4 more days the differentiated C2C12 cells had fused into myotubes. To induce insulin resistance in the differentiated skeletal muscle cells, the medium was replaced by lipid-containing medium. The latter was prepared by addition of FFA (palmitate dissolved in 0.1 M NaOH) to DMEM containing 2% fatty acid-free BSA. Myotubes were incubated for 16 h in the above medium in the presence or absence of 0.75 mM palmitate. To exclude the possibility that any FFA can induce insulin resistance, 0.75 mM oleic acid was also added to myotubes and served as negative control (data not shown).

Determination of glucose uptake by differentiated C2C12 skeletal muscle cells

Following induction of insulin resistance, all culture medium was removed from each well and replaced with 1 ml of fresh culture medium in the absence or presence of 10 μM fluorescent 2-NBDG (Molecular Probes-Invitrogen, CA/Molecular Probes, Eugene, OR), a new fluorescent derivative of glucose with a 2-[7-nitrobenza-2-oxa-1,3-diazol-4-yl] amino group at the C-2 position [33]. For this purpose, the cells were incubated at 37°C with 5% CO₂ for 1 h. The cells were then washed twice with cold phosphate-buffered saline (PBS) and collected for flow cytometric measurement.

Transfection with dominant-negative Ras

To block Ras we transfected differentiated C2C12 cells with 2 µg of green fluorescent protein plasmid (pGFP) or dominant-negative (DN) GFP-Ras (17N), using lipofectamine 2000 reagent according to manufacture’s instructions (Invitrogen, Carlsbad, CA, USA). At 48 h post-transfection the cells were subjected to induction of insulin resistance, as described above. The cells were then either harvested and analyzed by Western blotting or tested for glucose uptake using the 2-NBDG method described above.

Western blotting

To examine the impact of Ras inhibition on IκB and NF-κB, we performed Western immunoblotting with specific antibodies. Muscle and fat lysates were obtained from HF-induced mice, subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), and Western blotted as previously described [30] with one of the following antibodies: anti-IκB, anti-p-IκB, anti-NF-κB (Santa Cruz Biotechnology, Santa Cruz, CA) or anti-tubulin (eBioScience, San Diego, CA).

The levels of Ras GTP were determined as described previously [30]. Protein bands were visualized with an enhanced chemiluminescence kit (Amersham Pharmacia Biotech, Arlington Heights, IL) and quantified by densitometry with Image EZQuant-Gel software®.

Glut4 expression determined by reverse transcription–PCR

RNA was extracted from 10⁶ C2C12 muscle cells using an RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. Reverse transcription (RT)–PCR was performed according to the protocol of the Reverse-IT™ 1st Strand Synthesis Kit (ABgene, Epsom, UK). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was analyzed using the following primers: GAPDH forward 5'-GCTGAGCCAGTACCAG-3' and GAPDH reverse 5'-TCCACGACCCCTGTTG-CCTGTA-3'.
of 2-NBDG. Two hours later, muscle, liver and fat tissues were removed and single-cell suspensions from each of those tissues were tested by fluorescence activated cell sorting (FACS) for the presence of 2-NBDG. Mice in a control group ($n=8$) were similarly injected and treated, except that they were injected with pGFP instead of pDN-Ras-GFP.

We produced a model for type 2 diabetes by feeding a high-fat diet (TD.06415, Harlan Laboratories, Madison, WI, USA) to male
C57Bl/6 mice, starting when they were 6 weeks old as detailed earlier [34]. These high-fat-induced diabetic mice were treated orally either with 30 mg/kg of F-FTS (n = 5) or, as a control, with carboxymethyl cellulose (CMC) vehicle (n = 5). Thirteen weeks later, the mice were injected i.v. with 500 μg of 2-NBDG. Two hours after injection, muscle and liver tissues were removed and analyzed for glucose uptake as described above.

Ras inhibition and effect on an in-vivo model of type 2 diabetes

To study the effect of F-FTS on type 2 diabetes in high-fat diabetic mice, we started to treat them, at the same time as the high-fat diet was initiated, with five different daily treatments, as follows: F-FTS (20 mg/kg body weight), injected intraperitoneally (i.p.; n = 30); F-FTS (30 mg/kg body weight), p.o. (p.o.; n = 10); FTS (60 mg/kg body weight), i.p. (n = 10); CMC (control), i.p. (n = 10); or PBS (control), i.p. (n = 30). Mice were considered diabetic when glucose uptake (Fig.3C).

Testing of the above tissues for Ras-GTP expression revealed a significant increase in fluorescent glucose uptake in these tissues (an increase of 214% ± 6% relative to the GFP-treated controls, respectively) (Fig. 2E and F). On the other hand, the expression of its inhibitor, IkB, was significantly higher both in its total level and in its phosphorylated form (p-IkB) by 160% ± 14% and 369% ± 53%, respectively in the presence of F-FTS compared to control (Fig. 2E and F). These findings are consistent with the results obtained by treatment with DN-Ras.

F-FTS treated mice exhibit increased glucose uptake accompanied by altered IkB/NF-κB expression

To verify the above in vivo findings in a relevant type 2 diabetes model, we treated 6-week-old C57Bl/6 mice fed on a high fat diet with F-FTS or, as a control, with PBS for 13 weeks, as described above, and then examined the ability of their muscle, fat and liver tissues to absorb intravenously injected fluorescent glucose. Cells obtained from the muscle and liver tissues of F-FTS-treated mice exhibited a significant increase in fluorescent glucose uptake compared to control (176 ± 18% and 153 ± 7%, respectively; Fig. 4A, B). No significant differences in glucose uptake were observed in the fat tissues (data not shown).

Immunoblot assays for IkB and NF-κB expression showed increased IkB expression compared to controls in muscle (by 158 ± 10%) and fat (by 181 ± 13%) tissues obtained from F-FTS treated mice, as opposed to a significant decrease compared to controls in NF-κB expression by 30 ± 10% and by 38 ± 8%, respectively (Figure 4C, D). No differences were found in the liver tissue (data not shown). Taken together, these results showed that Ras inhibition caused an increase in IkB/NF-κB-dependent glucose uptake in vivo.
Ras Inhibition Induces Glucose Uptake

A. PBS → F-FTS 20 ip

% Diabetic mice vs. time (days)

B. Glucose concentration (mg/dl)

F-FTS 20 ip vs. PBS

C. CMC → F-FTS 30 po

% Diabetic mice vs. time (days)

D. Glucose concentration (mg/dl)

FTS vs. F-FTS 30 and 60 po

E. % Weight gain vs. time (weeks)

F. Insulin concentration (ng/ml)

PBS vs. F-FTS ip

G. Insulin concentration (ng/ml)

CMC vs. F-FTS 30 and 60 po
Consistent with these findings, we showed here for the first time that inhibition of Ras by DN-Ras or F-FTS, promoted anti-inflammatory response in a muscle cell line and in mouse tissues. This study is the first to show a clear association between Ras signaling and insulin resistance in muscle, fat and liver. We found that inhibition of Ras activation by transfection with DN-Ras or by treatment with the small-molecule Ras inhibitor F-FTS induced glucose uptake in vivo, indicating higher insulin sensitivity. In addition, we demonstrated that inhibition of Ras in vivo by hydrodynamic injection of DN-Ras or by daily treatment with F-FTS in an experimental murine model of HF-induced diabetes resulted in similar findings of increased uptake of fluorescently labeled glucose by muscle, fat and liver tissues.

To characterize the signaling pathway by which Ras inhibition promotes insulin sensitivity, we studied the expression of key regulators known to participate in insulin-signaling pathways. For example, activation of the IκB/NF-κB cascade activates a widespread proinflammatory program. IκB kinase (IKK) phosphorylates certain serine residues on insulin receptor kinase 1 that lead to degradation of the receptor, thereby decreasing insulin signaling.

Activated Ras plays an important role in modulating a number of signaling molecules that trigger cell proliferation, differentiation, and survival [37,38]. These observations are in line with several studies showing that Ras inhibition attenuates inflammatory responses in experimental models [25,31,39,40]. Resistance to insulin, resulting in decreased glucose uptake, is a major factor contributing to the development of type 2 diabetes [41]. The mechanisms responsible for inducing resistance to insulin are not completely understood, but accumulating data point to a robust association between insulin resistance and inflammation. Obesity promotes insulin resistance by resulting in a state of chronic inflammation that involves production of proinflammatory cytokines (TNF-α, IL-6), an increase in the number of macrophages, and activation of a complex cascade of signaling events in muscle, fat and liver tissues [6,35,42]. Consistent with these findings, we showed here for the first time that inhibition of Ras by DN-Ras or F-FTS, promoted anti-inflammatory response in a muscle cell line and in mouse tissues.
(IRS-1), leading to impairment of insulin signal transduction. In addition, the IKK signaling pathway is upregulated and activated, both in insulin-resistant humans and in rodent skeletal muscles [43]. Increased expression of IKK results in inhibition of IkB and activation of NF-κB; the latter subsequently transcriptionally activates a set of inflammatory pathway genes that induce resistance to insulin (see scheme, Fig. 6)[44].

Based on the above knowledge, we sought to explore the influence of Ras inhibition on the IkB/NF-κB cascade in a conventional model of insulin resistance. We found that Ras inhibition led to an increase in IkB, which inhibited the expression of NF-κB both in vitro and in vivo (Figs. 1–4). The improvement in glucose uptake in liver tissue of the F-FTS treated animals was not correlated with increased expression of IkB (Fig 4). This finding could result from the long period (13 weeks) of treatment that may influence the duration of the increased IkB expression. Overall, the observation that insulin resistance was attenuated by Ras inhibition in association with regulation of IkB and NF-κB provides a possible link between Ras, inflammation, and negative regulation of insulin signaling.

The most downstream factor in the insulin cascade is Glut4, an essential transporter responsible for translocation of insulin-regulated glucose into the cell [45]. We therefore examined the effect of Ras inhibition on Glut4 mRNA levels in insulin-resistant C2C12 myotubes treated with F-FTS. We found an increase in Glut4 mRNA levels after F-FTS treatment. These results suggested that the higher sensitivity to insulin was attributable to Ras inhibition, which may be related to the increase in expression of Glut4 transporter in the plasma membrane and the subsequent potentiated influx of glucose into the cell (see scheme, Figure 6). Taken together, our results suggest dual affects of Ras on insulin sensitivity and glucose uptake via two distinct pathways (Figure 6).

Previous studies have shown that both FTS and the small synthetic molecule F-FTS act primarily by inhibiting active Ras proteins and are mimicked by dominant negative Ras [30,46]. Therefore, mice fed a high-fat diet and concomitantly treated with F-FTS may serve as an appropriate in vivo model for examining the effect of Ras inhibition on an experimental model of type 2 diabetes. Our results showed that treatment with either FTS or F-FTS significantly attenuated the incidence of hyperglycemia in this model. The potential contribution of Ras-mediated insulin sensitization in this in vivo model is supported by the finding that circulating insulin levels were decreased in the FTS-treated mice (Fig. 5). The observed decrease of insulin level most likely resulted from the increased uptake of glucose into the tissues but could also be caused by a direct effect on the pancreas. Further studies should be performed to clarify the cause of the decrease in serum insulin levels.

Taken together, the results of this study showed that inhibition of Ras signaling enhances both insulin sensitivity and glucose uptake in vitro and in vivo. These observations were corroborated by the beneficial effects of Ras inhibition that resulted in attenuation of hyperglycemia in a conventional type 2 diabetes model. It should be noted, however, that Ras inhibition may modify inflammatory responses in other tissues as well. These findings pave the way for a novel approach to the potential treatment of insulin resistance and type 2 diabetes.

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Author Contributions

Conceived and designed the experiments: AM EA JG YK. Performed the experiments: AM EA. Analyzed the data: AM EA JG YK. Contributed reagents/materials/analysis tools: AM EA JG YK. Wrote the paper: AM EA JG YK.

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