Control of hyperglycemia in male mice by leflunomide: mechanisms of action

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Abstract

p70 S6 kinase (S6K1) is a serine/threonine kinase that phosphorylates the insulin receptor substrate-1 (IRS-1) at serine 1101 and desensitizes insulin receptor signaling. S6K1 hyperactivation due to overnutrition leads to hyperglycemia and type 2 diabetes. Our recent study showed that A77 1726, the active metabolite of the anti-rheumatoid arthritis (RA) drug leflunomide, is an inhibitor of S6K1. Whether leflunomide can control hyperglycemia and sensitize the insulin receptor has not been tested. Here we report that A77 1726 increased AKT S473/T308 and S6K1 T389 phosphorylation but decreased S6 S235/236 and IRS-1 S1101 phosphorylation in 3T3-L1 adipocytes, C2C12 and L6 myotubes. A77 1726 increased insulin receptor tyrosine phosphorylation and binding of the p85 subunit of the PI-3 kinase to IRS-1. A77 1726 enhanced insulin-stimulated glucose uptake in L6 myotubes and 3T3-L1 adipocytes, and enhanced insulin-stimulated glucose transporter type 4 (GLUT4) translocation to the plasma membrane of L6 cells. Finally, we investigated the anti-hyperglycemic effect of leflunomide on ob/ob and high-fat diet (HFD)-induced diabetes mouse models. Leflunomide treatment normalized blood glucose levels and overcame insulin resistance in glucose and insulin tolerance tests in ob/ob and HFD-fed mice but had no effect on mice fed a normal chow diet (NCD). Leflunomide treatment increased AKT S473/T308 phosphorylation in the fat and muscle of ob/ob mice but not in normal mice. Our results suggest that leflunomide sensitizes the insulin receptor by inhibiting S6K1 activity in vitro, and that leflunomide could be potentially useful for treating patients with both RA and diabetes.

Introduction

Type 2 diabetes is a major public health problem (Zimmet et al. 2001, 2014). Although many anti-diabetic medications are available, some have intolerable side-effects or lose their therapeutic efficacy after the long-term use (Nathan 2015). Failure to control hyperglycemia leads to diabetic complications, which account for most diabetes-related morbidity and mortality (Nathan 2015). Because of physical inactivity and
cTRING inflammatory cytokines, diabetic patients with rheumatoid arthritis (RA) may have worse hyperglycemia than those without RA (Herlitz-Cifuentes et al. 2015, Pinto et al. 2017). Individuals with RA have a significantly higher risk of developing type 2 diabetes (Jiang et al. 2015) and obesity (Versini et al. 2014) than those without RA. Currently, patients with both RA and diabetes are treated with antidiabetic and anti-RA drugs separately. A drug that controls both RA and hyperglycemia could greatly benefit patients with both problems.

The binding of insulin to its receptor activates the insulin receptor tyrosine kinase, leading to insulin receptor autophosphorylation and the phosphorylation of intracellular protein substrates such as the insulin receptor substrates (IRS) (Fig. 1B) (Guo 2013, 2014). Tyrosine-phosphorylated IRS interact with the p85 subunit of the PI-3 kinase and activate its catalytic p110 subunit (Fig. 1B). PI-3 kinase activation leads to serine phosphorylation and activation of the protein kinase B (AKT) (Guo 2013, 2014). AKT activation plays a critical role in glucose metabolism (Dann et al. 2007, Copps & White 2012). AKT activation stimulates glucose uptake by inducing translocation of the glucose transporter type 4 (GLUT4) to the plasma membrane of both adipose and muscle cells (Fig. 1) (Guo 2013, 2014). In addition, AKT also regulates glucose metabolism by stimulating glycolysis and inhibiting gluconeogenesis (Fig. 1B) (Guo 2013, 2014).

The mechanistic target of rapamycin (mTOR) kinase is a serine/threonine kinase activated by AKT. Overnutrition with high concentrations of amino acids and fatty acids also activates mTOR (Fig. 1B). p70 S6 kinase (S6K1), a serine/threonine protein kinase downstream of mTOR, phosphorylates the IRS and subsequently attenuates the activation of the PI3K pathway (Fig. 1B) (Fenton & Gout 2010). Constitutive S6K1 activation by hyperinsulinemia or overnutrition leads to insulin receptor desensitization (Boura-Hallon & Zick 2009, Copps & White 2012). S6K1 is also involved in regulating the expression of several energy expenditure-related genes such as the melanocortin-4 receptor (MC4R) (Um et al. 2004, 2006, Xia et al. 2012). S6K1−/− mice do not develop obesity and hyperglycemia when fed a high-fat diet (HFD) (Um et al. 2004). These mice have a significantly longer life span than the wild-type mice (Selman et al. 2009). Insulin receptor signaling is highly active in the metabolic tissues of the HFD-fed S6K1−/− mice, as evidenced by increased AKT phosphorylation in their liver, muscle and fat (Um et al. 2004). S6K1 is a key kinase driving insulin resistance and inducing obesity under conditions of nutrient overload (Dann et al. 2007).

Leflunomide is an orally administered prodrug proscribed for treating rheumatoid arthritis (RA) (Breedveld & Dayer 2000). Upon indigestion, it is rapidly and completely (>99%) converted in the gastrointestinal tract, plasma and liver to its active metabolite, A77 1726 (Fig. 1A) (Breedveld & Dayer 2000). Once in plasma, A77 1726 is avidly bound to plasma proteins, mainly albumin (Cannon & Kremer 2004). A77 1726 has a very long half-life of 15.5 days (range 14–18 days) and is cleared after it is metabolized into trifluoromethylenol-oxanilic acid (60–70%) and excreted into the urine (Breedveld & Dayer 2000, Cannon & Kremer 2004). A77 1726 is the only active metabolite of leflunomide and is solely responsible for its therapeutic activity. A77 1726 inhibits

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the activity of protein tyrosine kinases and dihydroorotate dehydrogenase (DHO-DHase) (Xu et al. 1995, 1996, 1997, 1999, Siemasko et al. 1996, 1998, Elder et al. 1997, Ruckemann et al. 1998). The ability of A77 1726 to inhibit DHO-DHase activity (the IC \(_{50}\) values of approximately 100 nM) is about 10–100 times stronger than its ability to inhibit the activity of protein tyrosine kinases such as p56 \(\text{lck}\), p59 \(\text{fyn}\) and the PDGF receptor (the IC \(_{50}\) values of approximately 25–50 µM) (Xu et al. 1995, 1996, 1999, Ruckemann et al. 1998). Inhibition of pyrimidine nucleotide synthesis by leflunomide was thought to be its primary mechanism of action (Williamson et al. 1996, Bruneau et al. 1998). However, addition of exogenous uridine, which normalizes pyrimidine nucleotide levels \(\text{in vitro}\) in cell cultures, only partially antagonizes this anti-proliferative effect. Co-administration of uridine with leflunomide in a lymphadenopathy and autoimmune disease model of MRL/MpJ-lpr/lpr mice and in a tumor xenograft model does not abrogate the immunosuppressive and antitumor activities of leflunomide (Xu et al. 1997, 1999). This suggests that leflunomide exerts its anti-proliferative and immunosuppressive activity by other mechanisms (Xu et al. 1997, 1999). Our recent study revealed that leflunomide and A77 1726 directly inhibit the activity of purified S6K1 in an \(\text{in vitro}\) kinase assay and inhibit the activity of S6K1 in cell culture, with an IC \(_{50}\) value of 50–75 µM (4-fold lower than its plasma levels in patients) (Doscas et al. 2014). Inhibition of S6K1 activity by A77 1726 leads to feedback activation of the PI-3 kinase pathway in tumor cell lines, as evidenced by increased AKT and S6K1 phosphorylation and decreased S6 phosphorylation (Doscas et al. 2014). Here, we report that A77 1726 increases S6K1 and AKT phosphorylation and stimulates GLUT4 translocation to the cell membrane and glucose uptake in myotubes and adipocytes (Fig. 1B). We further show that leflunomide controls hyperglycemia in ob/ob mice and in the mice with HFD-induced diabetes but not in normal mice.

**Materials and methods**

**Chemicals, antibodies and plasmid construct**

Leflunomide and A77 1726 were kindly provided by CinKate Corporation (Oak Park, IL, USA). Cytochalasin and rosiglitazone were purchased from Calbiochem (EMD Millipore). 3-isobutyl-1-methylxanthine (IBMX), carboxymethyl-cellulose sodium (CMC), uridine, dexamethasone and 2-deoxy-glucose (2-DG) were purchased from Sigma Aldrich. 2-DG (5–10 Ci (185–370 GBq)/mmol, 1mCi (37 MBq) was purchased from PerkinElmer. Insulin used in the \(\text{in vitro}\) study was purchased from Invitrogen (Life Technologies). Rapamycin (an mTOR inhibitor), antibodies against AKT, S6K1, S6, IRS-1, p85 of the PI-3 kinase and phospho-antibodies (AKTs473, AKT\(_{308}\), S6K1T389, S6S235/236, IRS-1S1101, IR\(_{1146}\) and IRS-1S636) were purchased from Cell Signaling Technology. Anti-β-actin monoclonal antibody was purchased from Santa Cruz Biotechnology. The sources of antibodies and their applications were listed in Table 1. mCherry-GLUT4- myc expression vector was kindly provided by Dr Amira Klip (The Hospital for Sick Children, Toronto, Ontario). Use of the radioactive isotope was approved by Rush University Medical Center. All methods were performed in accordance with the relevant guidelines and regulations of Rush University Medical Center and Yangzhou University.

**Cell lines and differentiation**

C2C12 (a murine myoblast cell line) and L6 cells (a rat myoblast cell line) were cultured in DMEM supplemented with 10% fetal bovine serum. For myotube differentiation, the confluent monolayers of C2C12 cells were cultured in DMEM containing 10% horse serum for 2 weeks during which the same fresh medium was replenished every two days. For L6 myotube differentiation, the cells were cultured in DMEM containing 2% calf serum for two weeks. The early passages of 3T3-L1 adipocytes (within 15 passages) were differentiated according to a detailed protocol of Zebisch et al. (2012). After incubation in insulin-free medium for 7–14 days, more than >95% of the cells exhibited an adipocyte-like phenotype. All three cell lines were purchased from the American Type Culture Collection (ATCC).

**Glucose uptake**

C2C12 myotubes express very low levels of GLUT4 and therefore were not used for glucose uptake experiments. Differentiated 3T3-L1 adipocytes and L6 myotubes were incubated overnight in insulin-free DMEM medium. Cells were starved of serum for 4 h and then incubated in the absence or presence of A77 1726 (200 µM) or rapamycin (20 nM) for 1 h without or with 2x amino acids in essential balanced salt solution (EBSS) for another 1 h. Cells were left unstimulated or stimulated with 20 nM insulin for 45 min. Unlabeled 2-DG (0.1 mM) and [\(^{3}H\)]-DG were added to the cells in the KRP-HEPES buffer (10 mM HEPES, pH 7.4, 131.2 mM NaCl, 4.7 mM KCl, 1.2 mM...
Table 1  Antibodies information.

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<th>Manufacturer, catalog #, and/or name of individual providing the antibody</th>
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MgSO₄, 2.65 mM CaCl₂, 2.5 mM NaH₂PO₄, and 1% bovine serum albumin) at 37°C for 5 min. The reaction was terminated by adding 10 µM cytochalasin B followed by wash with ice-cold PBS three times. The cells were lysed in 0.2 M NaOH. The radioactivity was measured in a liquid scintillation counter. Non-specific 2-DG uptake was measured by adding cytochalasin B (10 µM) into the cells prior to the addition of 2-DG. The values of 2-DG uptake, after correction by subtracting the value of the non-specific 2-DG uptake, were normalized by protein concentrations, which were quantified by using a Bio-Rad Protein Assay kit (Bio-Rad).

**Western blot**

C2C12 myotubes grown in 6-well plates were starved of serum for 4 h. A77 1726 or rapamycin was added and incubated for 2 h. Cells were left unstimulated or stimulated with 20 nM insulin for 20 min. Differentiated 3T3-L1 adipocytes and L6 myotubes were treated as described in the glucose uptake experiments. Cell lysates were prepared in NP-40 lysis buffer (50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 1% NP-40, 5 mM EDTA, 10 µg/mL aprotinin, 10 µg/mL leupeptin, and 1 mM phenylmethysulfonyl fluoride, 2 mM sodium vanadate) and analyzed for protein phosphorylation with the indicated antibodies, followed by probing with antibodies against total proteins. The density of the bands was analyzed by using NIH ImageJ software and normalized by the arbitrary units of their corresponding total proteins. Quantified results were presented as the mean±standard deviation (s.d.) from three experiments (Figs 2, 3, 4 and 5) in bar graphs.

**Immunoprecipitation**

L6 myotubes and 3T3-L1 adipocytes were first starved of serum for 2 h or overnight, respectively, then incubated either in an amino-acid free medium (EBSS) or EBSS medium containing 2× amino acids (2×AA) in the absence or presence of A77 1726 for 5 h. Cells were left unstimulated or stimulated with insulin (100 nM) for 10 min. Cell lysates were immunoprecipitated with a rabbit monoclonal antibody against the p85 subunit of the PI-3 kinase, followed by probing with anti-p85 and anti-IRS1 antibodies in Western blot.

**Confocal microscopy**

Undifferentiated L6 cells seeded on coverslips were transiently transfected with mCherry-GLUT4-myc expression vector DNA using FuGENE6 following the manufacturer’s protocol. After incubation for 24 h, the cells were starved of serum for 2 h and then incubated in the absence or presence of A77 1726 (200 µM) for 4 h without or with 2× the amino acid concentrations in EBSS. Cells were left unstimulated or stimulated with 100 nM insulin for 30 min. The coverslips were collected,
fixed and mounted with 50% glycerin in PBS containing 4,6-diamidino-2-phenylindole (DAPI) (0.5 µg/mL; Sigma Chemical). mCherry-tagged GLUT4 fluorescence was visualized under a Leica LP8 confocal microscope. The percent of cells positive for GLUT4 translocation into the plasma membrane among total mCherry-GLUT4-expressing cells was calculated by counting 10 randomly selected fields from each treatment. The results represent the mean ± standard deviation (s.d.) from one of three experiments with similar results.

Animals and drug administration

Use of animals and all experimental protocols were approved by the Institutional Animal Care and Use Committee of Rush University Medical Center and College of Veterinary Medicine, Yangzhou University. All mice were maintained on a 12-h light/darkness cycle and housed in ventilated cages at an ambient temperature of 23°C. Male ob/ob mice (B6.V-Lepob/0laHsd, male) were purchased from Harlan Laboratories, Inc. These mice were fed ad libitum on a normal chow diet (NCD). CMC (1.5% dissolved in distilled water) was used as a vehicle to prepare leflunomide. Mice (8–10-week-old) were given 1.5% CMC or leflunomide by gavage. Uridine was co-administered with leflunomide to ob/ob mice to determine whether leflunomide could still control hyperglycemia when pyrimidine nucleotide levels in various tissues were normalized. The uridine dose was based on our previous studies (Xu et al. 1997, 1999) that 2 g/kg, twice daily is sufficient to normalize or overshoot pyrimidine nucleotide levels in the fast proliferating tumor cells or lymphocytes of lpr/lpr mice. Uridine dissolved in saline was given by intraperitoneal (i.p.) injection. ob/ob mice were treated with the vehicle (1.5% CMC daily), leflunomide (35 mg/kg/day, daily, gavage), uridine (2 g/kg, twice daily, i.p.) or leflunomide (35 mg/kg/day, daily, gavage) + uridine (2 g/kg, twice daily, i.p.) for

Figure 2
Effect of A77 1726 on protein phosphorylation in the PI-3 kinase pathway. C2C12 myotubes were starved in serum-free medium for 4 h and then treated with the indicated concentrations of A77 1726 or rapamycin (50 nM) for 2 h. The cells were left unstimulated or stimulated with insulin (20 nM) for 20 min. Cells were harvested and analyzed for the phosphorylation of AKT (T308, S235/236, and S6K1 T389, S6K1 547, IRS-1 S636, IRS-1 S1101, IRS-1 S646, and IRS-1 S1101, IRS-1 S646, and S6K1 T389, S6K1 S235/236, followed by reprobing with their specific antibodies for total protein levels. Relative protein phosphorylation was determined by analyzing the density of bands and presented as bar graphs. The results are the mean ± standard deviation (s.d.) from three experiments. A77, A77 1726; *P < 0.05; **P < 0.01, compared to the insulin-stimulated control (no drug treatment).
Insulin receptor sensitization by leflunomide

Figure 3
A77 1726 sensitizes insulin receptor in L6 myotubes. This experiment and the remaining ones (Figs 4, 5, 6 and 7) were carried out under the condition of insulin resistance in which the cells were incubated in the presence of high concentrations of amino acids. L6 myotubes were first starved of serum for 4 h and then incubated either in an amino-acid free medium (EBSS) or EBSS medium containing 2× the amino acid concentrations (2×AA) found in MEM in the absence or presence of A77 1726 (200 µM) for 2 h. After stimulation with insulin (20 nM) for 20 min, cells are harvested and analyzed for the phosphorylation of AKT\(^{S473/T308}\) (A), S6K1\(^{T389/S235/236}\) (B), IRS-1\(^{S1101}\) (C), S6\(^{S240/244}\) (D), and reprobed with their specific antibodies for total proteins. Relative protein phosphorylation was analyzed by using an Image J software. The results are the mean ± s.e.m. from three experiments. A77, A77 1726; Rapa, rapamycin.

In vivo AKT activation

ob/ob mice were treated for three days. They were given a last dose on day 4 and then fasted for 6 h. Five minutes after insulin injection (2.5 unit/kg, intravenously), mice were sacrificed. Gastrocnemius muscle, mesenteric visceral white adipose and hepatic tissues (50–100 mg/sample) were collected and immediately homogenized in NP-40 lysis buffer. Protein concentrations were measured using a Pierce BCA Protein Assay kit (Thermo Fisher Scientific). AKT\(^{S473/T389}\), S6\(^{S240/244}\) and IRS-1\(^{S1101}\) phosphorylation was analyzed by Western blot. The density of the bands was analyzed by using NIH ImageJ software and normalized by the arbitrary units of their corresponding total proteins.

Statistical analysis

Data were presented as mean±standard deviation (S.D.) (glucose uptake and GLUT4 translocation assays) or standard error of the mean (S.E.M.) (blood glucose levels).
An unpaired Student t test was used to analyze the differences in glucose uptake in 3T3-L1 adipocytes and L6 myotubes in different groups, and the differences in the arbitrary number of Western blot data from the ImageJ analysis. Differences in blood glucose levels between different treatment groups were analyzed by repeated-measures ANOVA (analysis of variance). Differences in blood glucose levels in individual groups before and after treatment were statistically analyzed by a paired Student t test. A P value <0.05 was considered statistically significant. All statistics was performed with SigmaPlot 11 software (Systat Software, San Jose, CA, USA).

Results

A77 1726 induces feedback activation of the PI-3 kinase pathway

S6K1 inhibition by A77 1726 in tumor cell lines leads to feedback activation of the PI-3 kinase pathway through the insulin-like growth factor 1 (IGF-1) receptor (Doscas et al. 2014). Here, we tested if A77 1726 also induced feedback activation of the PI-3 kinase pathway in C2C12 myotubes. As shown in Fig. 2, insulin induced the phosphorylation of IRS-1S1101, AKT(T308), AKT(S473) and S6K1(T389) in C2C12 myotubes. A77 1726 enhanced insulin-induced phosphorylation of AKT(S473), AKT(T308) and S6K1(T389), both of which are phosphorylated by S6K1. In contrast, A77 1726 increased the phosphorylation of IRS-S636 (Fig. 2). This site is phosphorylated largely by mTOR. Of note, S6S235/236 and IRS-1S1101 were highly phosphorylated in unstimulated C2C12 myotubes. Rapamycin, an inhibitor of mTOR, increased AKT(T308) and AKT(S473) phosphorylation but inhibited IRS(S1101), IRS-S636, S6S235/236 and S6K1(T389) phosphorylation. Increased S6K1 phosphorylation by A77 1726 is consistent with previous observations with PF-4708671, a specific S6K1 inhibitor (Pearce et al. 2010, Shum et al. 2016).

A77 1726 enhances insulin receptor signaling

We next examined the effect of A77 1726 on protein phosphorylation in the PI-3 kinase pathway in L6 myotubes and 3T3-L1 adipocytes in the presence of high amino acid concentrations, a condition of insulin resistance. As shown in Fig. 3, insulin induced AKT(S473/T308) phosphorylation in the absence of amino acids in L6 myotubes. Consistent with previous observations (Patti et al. 1998), high amino
A77 1726 stimulates insulin receptor tyrosine phosphorylation and increases the insulin receptor substrate (IRS-1) binding to the p85α subunit of the PI-3 kinase in 3T3-L1 adipocytes and L6 myotubes cultured in the absence or presence of the p85 subunit of the PI-3 kinase at Y1146. A77 1726 increases the binding of the p85 subunit of the PI-3 kinase to IRS-1. 3T3-L1 adipocytes and L6 myotubes were treated in Fig. 3. Cell lysates were immunoprecipitated with an anti-p85 antibody followed by probing with anti-p85 and anti-IRS-1 antibodies in Western blot. Relative protein phosphorylation was analyzed by using Image J software. The results are the mean ± s.d. from three experiments. A77, A77 1726; *P<0.05; **P<0.01.

Um et al. (2004) reported that S6K1 deficiency leads to insulin receptor sensitization, as evidenced by increased insulin receptor tyrosine phosphorylation in the liver of insulin-treated mice, compared to that in wild-type mice. Here, we tested if A77 1726 could indeed enhance insulin receptor signaling. 3T3-L1 adipocytes and L6 myotubes preincubated with A77 1726 in the absence or presence of 2× amino acids and were left unstimulated or stimulated with insulin for 30 min. As shown in 5A, insulin-induced insulin receptor tyrosine phosphorylation in 3T3-L1 adipocytes and L6 myotubes that were cultured in the absence or presence of high concentrations of amino acids. This phosphorylation was further increased by A77 1726. Immunoprecipitation revealed that insulin stimulation increased the binding of the p85 subunit of the PI-3 kinase to IRS-1 in 3T3-L1 adipocytes and L6 myotubes cultured in the absence or presence of 2× amino acids. This binding was further increased by A77 1726 (Fig. 5B). Cell lysates were analyzed by Western blot. Equal levels of IRS-1 and the p85 subunit of the PI-3 kinase were found in the cell lysates used for immunoprecipitation (Fig. 5B).

A77 1726 increases GLUT4 translocation to the cell membrane

AKT activation induces the translocation of GLUT4 from the vesicles into the plasma membrane (Dann et al. 2007, Copps & White 2012). GLUT4 was detected in the cytoplasm of unstimulated L6 but was translocated to the plasma membrane in insulin-stimulated cells in the absence of amino acids (Fig. 6). A77 1726 alone slightly increased GLUT4 membrane translocation in L6 cells in the presence of 2× amino acids. Insulin also weakly induced GLUT4 translocation into the plasma membrane in the presence of 2× amino acids, almost as well as in the absence of 2× amino acids, probably due to the use of the undifferentiated L6 myoblast cells in this experiment. Alternatively, the relatively low transfection efficiency and a few GLUT4-positive cells under a high power field may shield the 2× amino acids-mediated inhibition of GLUT4 translocation. Nevertheless, A77 1726 significantly increased insulin-stimulated GLUT4 membrane translocation in L6 cells in the presence of 2× amino acid concentrations (Fig. 6).
A77 1726 increases glucose uptake

We next determined if insulin receptor sensitization by A77 1726 led to increased glucose uptake. Cells treated with high concentrations of amino acids were considered under the insulin resistance condition. Indeed, 2× the amino acid concentrations significantly decreased the basal level of glucose uptake in L6 myotubes by 29% and reduced insulin-stimulated glucose uptake by 22%, compared to their corresponding controls (Fig. 7A). A77 1726 increased insulin-stimulated glucose uptake in the presence of 2× the amino acid concentrations by 31%. Rapamycin included as a positive control increased glucose uptake by 19% (Fig. 7A) in insulin-stimulated L6 myotubes in the presence of 2× the amino acid concentrations. Slightly better stimulation of insulin-induced glucose uptake by A77 1726 was observed with 3T3-L1 adipocytes (Fig. 7B). Of note, A77 1726 is a cytostatic drug and does not affect the viability and proliferation of differentiated non-dividing cells. Cells were incubated in the presence of A77 1726 only for a few hours. Using an Enhanced Cell Counting Kit-8 (CCK-8), we found that A77 1726 (200 µM) did not have any cytotoxicity on 3T3-L1 adipocytes and L6 myotubes.
leflunomide treatment significantly lowered blood glucose levels ($P<0.01$). Treatment with uridine, a nucleoside used to normalize pyrimidine nucleotide levels in vitro and in vivo (Xu et al. 1997, 1999), had no effect on fasting blood glucose levels and did not block leflunomide-mediated control of hyperglycemia. These observations suggest that leflunomide controls hyperglycemia independent of its inhibitory effect on pyrimidine nucleotide synthesis. There were no significant differences in food intake and body weight among mice treated with CMC, leflunomide, uridine or leflunomide plus uridine for 3 days (Table 2).

We next conducted GTT to examine the ability of leflunomide to lower blood glucose levels in ob/ob mice. Blood glucose levels in mice receiving leflunomide or leflunomide plus uridine were significantly lower than those treated with CMC ($P<0.001$) (Fig. 8A). Uridine treatment alone did not significantly alter blood glucose levels in mice. ITT assay revealed that blood glucose levels were elevated in control mice or in mice treated with uridine 15 min after insulin injection (Fig. 8C). In contrast, insulin did not increase blood glucose levels in leflunomide-treated mice but was able to slightly increase blood glucose levels in mice treated with uridine plus leflunomide (Fig. 8C). Transient increase of blood glucose levels after insulin injection into the tails of control ob/ob mice is probably caused by stress or due to the insulin receptor internalization. Transient increase of blood glucose levels after insulin injection is consistent with observations made by others (He et al. 2013). UAC was significantly decreased in leflunomide- or leflunomide plus uridine-treated mice, compared to those treated with CMC or uridine alone (Fig. 8B and D) ($P<0.01$). Consistently, leflunomide treatment significantly decreased blood glucose levels in GTT (Fig. 8E and F) and ITT assays (Fig. 8G and H) in HFD-fed mice, compared to those treated with CMC. Leflunomide appears more effective in lowering blood glucose levels in ob/ob mice while only modestly lowering blood glucose levels in the mice with HFD-induced diabetes. Leflunomide treatment did not significantly alter blood glucose levels in mice fed a NCD (Fig. 8E, F, G and H).

Control of hyperglycemia by leflunomide

We first assessed the ability of leflunomide to control hyperglycemia in male mice since estrogen in female mice may protect against high-fat diet-induced metabolic syndrome. Female animals are prone to adipose tissue storage and glucose homeostasis, whereas males are predisposed to diabetes. As shown in Table 2, blood glucose levels were very high (>200 mg/dL) in ob/ob mice before treatment and remained very high in mice treated with CMC, a vehicle used to dissolve leflunomide. Fasting blood glucose levels decreased to normal levels in mice treated with leflunomide alone or with leflunomide plus uridine (<110 mg/dL). Statistical analysis revealed that
and leflunomide-treated mice were comparable (Fig. 9A and C) \((P>0.05)\). In contrast, insulin poorly induced AKTS473/T308 phosphorylation in the muscle, fat and liver of ob/ob mice (Fig. 9B). Densitometry analysis revealed that AKTS473/T308 phosphorylation in the insulin-sensitive tissue was not significantly different between the untreated and insulin-treated mice \((P>0.05)\) (Fig. 9D). Leflunomide treatment significantly increased insulin-stimulated AKTS473/T308 phosphorylation in the muscle and adipose tissues, compared to those mice treated with the control vehicle only \((P<0.05)\). AKTS473/T308 phosphorylation levels appeared to be higher in the liver of leflunomide-treated ob/ob mice than that of untreated ob/ob mice. However, statistical analysis did not reach significance. Similarly, AKTS473/T308 phosphorylation levels were also higher in the muscle, fat and liver of ob/ob mice treated with leflunomide plus uridine than those treated with uridine alone (Supplementary Fig. 1, see section on supplementary data given at the end of this article). However, there was no significant difference in S6 and IRS-1 phosphorylation in the muscle, fat and liver between untreated and leflunomide-treated mice (Supplementary Fig. 2).

**Discussion**

S6K1 hyperactivation has been implicated in insulin resistance and the development of hyperglycemia and obesity (Um et al. 2004, 2006). S6K1-deficient mice are resistant to HFD-induced hyperglycemia and obesity (Um et al. 2004, 2006). Shum et al. (2016) recently reported that PF-4708671, an S6K1 inhibitor, controls hyperglycemia in HFD-fed mice by sensitizing the insulin receptor. We recently identified S6K1 as a molecular target of leflunomide and A77 1726 (Doscas et al. 2014). Inhibition of S6K1 activity by A77 1726 in A375 melanoma cells leads to the feedback activation of the PI-3 kinase pathway, as evidenced by increased AKTS473 and decreased S6S235/S236 phosphorylation. Our present study showed that inhibition of S6K1 activity by A77 1726 led to the inhibition of S6S235/S236 and IRS-1'S1101 phosphorylation in mouse C2C12 and rat L6 myotubes and in mouse 3T3-L1 adipocytes under normal and/or insulin resistance conditions. A77 1726 enhanced insulin-induced insulin receptor tyrosine phosphorylation, binding of the p85 subunit of the PI-3 kinase to IRS-1 (Fig. 5), and insulin-induced GLUT4 translocation to the plasma membrane in L6 cells in the presence of high amino acid concentrations (Fig. 6). Leflunomide treatment increased AKTS308/S473 phosphorylation in the muscular and adipose tissues.
In contrast, leflunomide treatment decreased blood glucose levels in NCD-fed lean mice treated with CMC or leflunomide (35 mg/kg/day) for 3 days and then evaluated for glucose levels 2 h after glucose injection. The arbitrary values of AUC were calculated and shown in a bar graph. **CMC vs leflunomide, **CMC vs leflunomide + uridine, P < 0.001; leflunomide vs leflunomide + uridine, P = 0.002. Lef, leflunomide; Uri, uridine. The arbitrary values of AUC were calculated and shown in a bar graph. **CMC vs leflunomide or leflunomide + uridine, P = 0.002. Leflunomide did not inhibit S6 phosphorylation in vivo. These observations collectively suggest that inhibition of S6K1 by A77 1726 is primarily responsible for its anti-hyperglycemic effect. Guo et al. (1997) reported earlier that chronic use of leflunomide has no effect on blood glucose levels in healthy rats. Because there is no insulin resistance in the insulin-sensitive tissues of normal animals, leflunomide cannot further sensitize the insulin receptor. Consistently, we found that there were no significant differences in blood glucose levels in NCD-fed lean mice treated with CMC or leflunomide (Fig. 8E, F, G and H).

We investigated the inhibitory effect of A77 1726 on the phosphorylation of two relevant substrates of S6K1, S6235/236 and IRS-1S1101. Due to feedback activation of the PI-3 kinase pathway, S6K1 phosphorylation was increased in A77 1726-treated cells. A77 1726 inhibited S6235/236 and IRS-1S1101 phosphorylation in myotubes and adipocytes in vitro. In contrast, leflunomide treatment did not significantly decrease S6235/236 and IRS-1S1101 in the metabolic tissues of insulin-stimulated mice in vivo (Supplementary Fig. 2). Similar to our observations, Shum et al. (2016) reported that PF-4708671, a more potent inhibitor of S6K1 than A77 1726, did not inhibit S6 phosphorylation in vivo. These investigators suggest that compensatory S6K2 activation may shield the inhibitory effect of PF-4708671 on S6 phosphorylation (Shum et al. 2016). Pearce et al. (2010) reported that PF-4708671 did not inhibit early S6 phosphorylation in 293 cells in the presence of serum. We speculate that inhibition of S6K1 activity by A77 1726 or PF-4708671 may initially inhibit S6 and IRS-1 phosphorylation. However, when S6K1 is further feedback activated, incomplete inhibition of S6 activity may account for lack of inhibition of S6 and IRS-1 phosphorylation. Within a few minutes after insulin injection, leaked S6K1 activity is sufficient to fully phosphorylate S6 and IRS-1. However, once AKT is feedback activated, AKT phosphorylation levels will remain high for a while. Activated AKT continues to regulate glucose metabolism until it is dephosphorylated and inactivated. IRS-1 is phosphorylated at S636 by mTOR (Copps & White 2012). Our present study showed that due to feedback activation of mTOR, A77 1726 induced IRS-1S636 phosphorylation in C2C12 myotubes. Interestingly, IRS-1S636 phosphorylation by ROCK1 leads to the activation of PI-3 kinase (Copps & White 2012). Thus, A77 1726 may improve insulin receptor signaling by inhibiting IRS-1S1101 and by increasing IRS-1S636 phosphorylation.
Leflunomide and A77 1726 inhibit S6K1 activity with the IC<sub>50</sub> values of approximately 50–75 µM (Doscas et al. 2014). Plasma concentrations of A77 1726 in RA patients treated with leflunomide (20 mg/day) are higher than 200 µM (Chan et al. 2005). A77 1726 in the blood of mice treated with leflunomide at a dose of 35 mg/kg had a remarkably long half-life of 15 h. The blood concentrations of A77 1726 reached a peak of 500 µM within 4 h and remained at 250 µM at 24 h after a single dose of 35 mg/kg of leflunomide in mice (Chong et al. 1999). These data suggest that A77 1726 concentrations in plasma are high enough to inhibit S6K1 activity. Indeed, AKT phosphorylation was increased in the metabolic tissues of leflunomide-treated mice. Blood glucose levels were decreased to normal levels in ob/ob mice treated with leflunomide at a dose of 35 mg/kg/day. Co-administration with uridine was unable to block the anti-glycemic effect of leflunomide. An earlier clinical study revealed that the mean glucose levels in the blood of RA patients treated with leflunomide (83 mg/dL) were significantly lower than those treated with other regimens (93 mg/dL) (Rho et al. 2009). About 10% of patients treated with leflunomide undergo weight loss that cannot be attributed to diarrhea or other gastrointestinal side effects and is likely due to an increased metabolic rate (Coblyn et al. 2001). These observations collectively suggest that A77 1726 concentrations are high enough to inhibit S6K1 activity, subsequently leading to better control of hyperglycemia. Inflammatory cytokines, in particular TNF-α, can also desensitize the insulin receptor and contribute to the development of hyperglycemia (Copps & White 2012). Anti-TNF-α therapy does not lower blood glucose levels in RA patients (Rovensingh et al. 2007). Many anti-inflammatory drugs, unlike leflunomide, do not lower blood glucose levels (Rho et al. 2009), suggesting that inhibition of inflammation is not a sufficient explanation for better control of hyperglycemia.

We are aware of several weaknesses in our study. First, the evidence supporting the conclusion that leflunomide sensitized the insulin receptor and controlled
hyperglycemia by inhibiting the activity of S6K1 in vivo is weak. Increased AKT phosphorylation in the tissue of leflunomide-treated mice with insulin resistance indirectly suggests feedback activation of the PI-3 kinase pathway due to the inhibition of S6K1 activity. However, inhibition of S6 and IRS-1 phosphorylation was not seen in the tissue of leflunomide-treated animals. This is consistent with the observations in a study using PF-4708671, a potent S6K1 inhibitor (Shum et al. 2016). Second, leflunomide worked extremely well in lowering blood glucose levels in the ob/ob mice, even with a relatively small sample size, the P values reached statistically significant levels. However, leflunomide only modestly decreased blood glucose levels in the GTT and ITT in the mouse model of the HFD-induced diabetes. Third, leflunomide treatment significantly enhanced insulin-stimulated AKT phosphorylation in the muscle and adipose tissues of ob/ob mice. Leflunomide treatment increased AKT phosphorylation in the liver of ob/ob mice, but this was not statistically significant due to a relatively small number of animals and the high variability of AKT phosphorylation. Fourth, since there are already many anti-diabetic drugs available for treating hyperglycemia, leflunomide might be only useful for a certain type of patient population such as those with both diabetes and RA. The diabetes mouse models used in our study do not have RA, thus not completely recapitulating a clinical setting in which leflunomide deems to be used.

Chronic use of rapamycin does not control but rather exacerbates hyperglycemia in mouse models and in patients (Di Paolo et al. 2006, Schindler et al. 2014). Failure of rapamycin to control hyperglycemia is, after chronic use, due to its ability to inhibit the activity of mTORC2 (Fig. 1B), which is responsible for phosphorylating AKT(S473) (Lamming et al. 2012). In the present study, we did not follow-up the anti-hyperglycemic effect after chronic use of leflunomide. However, several lines of evidence suggest that chronic use of leflunomide will likely maintain its therapeutic efficacy: (1) Unlike rapamycin, A77 1726 inhibits S6K1 (Doscas et al. 2014), a kinase downstream of mTOR. Therefore, A77 1726 does not act like rapamycin to inhibit mTORC2 (Fig. 1B); (2) The anti-hyperglycemic effect of PF-4708671 after chronic use is sustainable in vivo (Shum et al. 2016). Both A77 1726 and PF-4708671 control hyperglycemia by inhibiting S6K1; (3) Blood glucose levels are significantly lower in RA patients chronically treated with leflunomide than those treated with other drugs (Rho et al. 2009). Nevertheless, whether leflunomide can maintain a long-lasting therapeutic effect after chronic use should be verified first in animal models before it can be investigated in patients.

In summary, our present study showed that A77 1726 induced feedback activation of the PI-3 kinase pathway by inhibiting S6K1 activity and that A77 1726 increased glucose uptake and GLUT4 translocation to the plasma membrane in vitro (Fig. 1B). A77 1726 may also improve glucose metabolisms by stimulating glycogen synthesis and by inhibiting gluconeogenesis through activated AKT (Fig. 1B). Leflunomide was capable of controlling hyperglycemia and improving insulin sensitivity in vivo. Leflunomide could be particularly useful for treating RA patients who also have type 2 diabetes.

Supplementary data
This is linked to the online version of the paper at https://doi.org/10.1530/JOE-17-0536.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement
X X conceived concept, wrote manuscript and researched data. J C; J S, M E D, J Y; A J W conducted experiments and contributed to discussion; Y L (University of Chicago) contributed to discussion; Y L (Baylor College of Medicine) R A P contributed to discussion, reviewed and edited the manuscript.

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Insulin receptor sensitization by leflunomide

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Insulin receptor sensitization by leflunomide

J Chen et al.

58


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