Insulin-regulated aminopeptidase inhibitors do not alter glucose handling in normal and diabetic rats

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Abstract

Insulin-regulated aminopeptidase (IRAP) co-localizes with the glucose transporter 4 (GLUT4) in GLUT4 storage vesicles (GSV) in insulin-responsive cells. In response to insulin, IRAP is the only transmembrane enzyme known to translocate together with GLUT4 to the plasma membrane in adipocytes and muscle cells. Although the intracellular region of IRAP is associated with GLUT4 vesicle trafficking, the role of the aminopeptidase activity in insulin-responsive cells has not been elucidated. The aim of this study was to investigate whether the inhibition of the aminopeptidase activity of IRAP facilitates glucose uptake in insulin-responsive cells. In both *in vitro* and *in vivo* studies, inhibition of IRAP aminopeptidase activity with the specific inhibitor, HFI-419, did not modulate glucose uptake. IRAP inhibition in the L6GLUT4myc cell line did not alter glucose uptake in both basal and insulin-stimulated state. In keeping with these results, HFI419 did not affect peripheral, whole-body glucose handling after an oral glucose challenge, neither in normal rats nor in the streptozotocin (STZ)-induced experimental rat model of diabetes mellitus (DM). Therefore, acute inhibition of IRAP aminopeptidase activity does not affect glucose homeostasis.

Key Words

- ▶ IRAP
- ▶ aminopeptidase
- ▶ glucose
- ▶ GLUT4
- ▶ diabetes

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Introduction

Insulin-regulated aminopeptidase (IRAP) is a marker protein for specialized vesicles containing the insulin-responsive glucose transporter 4 (GLUT4) (Keller *et al.* 1995). IRAP is localized in distinct organelles within insulin-dependent cells, including sorting and recycling endosomes, the endosomal-trans-Golgi network (TGN) and GLUT4 storage vesicles (GSVs) (Bryant *et al.* 2002).

IRAP is a type II integral membrane protein of 160 kDa and a member of the M1 aminopeptidase family (Keller *et al.* 1995). IRAP has two major functional domains: the carboxy terminal catalytic domain, containing the GAMEN motif (amino acids 428–432)

and the HEXXH(X)₁₈E Zn²⁺-binding motif (amino acids 464–487) and a highly conserved intracellular amino terminal trafficking domain. In insulin-responsive cells, the intracellular domain is found to bind to a number of proteins, including AS160, and demonstrated to modulate insulin-dependent GSV tethering and trafficking (Ross *et al.* 1996), reviewed in Albiston and coworkers (Albiston *et al.* 2007).

In the basal state, IRAP occurs predominantly in intracellular vesicles where the catalytic site is intraluminal. Upon insulin stimulation, IRAP translocates to the plasma membrane with GLUT4, where the catalytic

site is exteriorized. The functional importance of the aminopeptidase activity in relation to glucose uptake in insulin-responsive cells has not been elucidated. We have previously demonstrated that two structurally distinct classes of IRAP inhibitors, the peptide-based angiotensin IV and LVV hemorphin-7 and the benzopyran-based HFI-419, all enhanced glucose uptake in hippocampal neurons depolarized with either potassium or cAMP (Albiston *et al.* 2008, Fernando *et al.* 2008). However, in the basal state, IRAP inhibitors did not alter glucose uptake in hippocampal neurons, nor were we able to demonstrate an insulin-stimulated glucose uptake (Albiston *et al.* 2008, Fernando *et al.* 2008). To delineate the potential role of the aminopeptidase activity of IRAP on insulin-dependent glucose uptake, we utilized the well-characterized insulin-responsive muscle cell line L6GLUT4myc. In this study, we investigated whether the IRAP inhibitor, HFI-419, modulated glucose uptake in either the basal or insulin-stimulated L6GLUT4myc cells. Consistent with other insulin-responsive cells, in L6 cells, IRAP is found predominantly in GLUT4 vesicles and translocates with GLUT4 to the plasma membrane after insulin stimulation (Urso *et al.* 1999, Li *et al.* 2016). In addition, we investigated the effect of this inhibitor on glucose clearance *in vivo* in normal and streptozotocin (STZ)-induced diabetes mellitus (DM) rats after an oral glucose challenge.

Materials and methods

Materials

L-Leucine-4-methyl-7-coumarinylamide (Leu-MCA) (Sigma-Aldrich). IRAP inhibitor, HFI-419, was dissolved in dimethyl sulfoxide (DMSO, Calbiochem). For animal studies, HFI-419 was first dissolved in 100% DMSO, and then diluted 1:10 in 30% (2-hydroxypropyl)- β -cyclodextrin (HPBCD; Sigma-Aldrich) to the final concentration. Streptozotocin was purchased from ICN Biomedicals Inc., Ohio. Actrapid insulin (100 IU/mL) was purchased from Novo Nordisk Pharmaceuticals Ltd. L6GLUT4myc cells were used between passages 14 and 16 and were a generous gift from Prof. A Klip (Patel *et al.* 2003).

Animals

Adult, male, Sprague–Dawley rats were purchased from the Animal Resource Centre (Western Australia) and

housed on a 12-h light/darkness cycle with *ad libitum* access to standard rat chow and water. All intramuscular (IM) injections were administered via caudal thigh muscle and all intravenous (IV) injections were administered via tail vein. All animal experiments were approved by the Howard Florey Institute Animal Ethics Committee.

Measurement of radiolabeled 2-deoxyglucose uptake

L6GLUT4myc cells were maintained in Dulbecco Modified Eagles Medium (DMEM) and 10% fetal bovine serum (FBS) and differentiated in DMEM and 2% FBS as described previously (Patel *et al.* 2003). Cells were used between days 8 and 10 after addition of differentiation media. Glucose uptake assays were performed using the d-2-deoxy-d-[2,6- ^3H]glucose (^3H -2DG) as described previously (Yamamoto *et al.* 2011). In brief, cells were incubated in FBS-free medium for 24 h, and then incubated in Krebs–Ringer–HEPES (KRH) buffer for 30 min with human insulin (Sigma-Aldrich) (0, 25 and 100 nM) plus or minus HFI-419 IRAP inhibitor (10^{-6}M and 10^{-7}M), followed by addition of 5 mM 2DG and 0.5 μCi /well of radiolabeled 2DG and a further 10-min incubation. For each assay, two wells were prepared with the inclusion of cytochalasin B (20 μM) to determine the nonspecific glucose uptake. For the 24-h incubations with HFI-419, the inhibitor was added just after the change to FBS-free serum media the day before the glucose uptake assay was performed.

Western blot analysis

Cell lysates (30 μg) were prepared and resolved on SDS–PAGE followed by Western blotting for IRAP as previously described (Fernando *et al.* 2007). Blots were stripped and re-probed using the rabbit anti-GLUT4 (Abcam).

Kinetic analysis of IRAP inhibition

Recombinant IRAP was prepared, and kinetic analysis was performed as previously described except that the fluorescent substrate l-leucine-4-methyl-7-coumarinylamide (Leu-MCA) was utilized (Lew *et al.* 2003a). Linear regression analysis of kinetic data expressed in double-reciprocal (Lineweaver–Burk) form resulted in *R* values greater than 0.99 for each curve. Inhibitory constant (K_i) for HFI-419 was calculated from the relationship $\text{IC}_{50} = K_i (1 + (S)/K_m)$, where K_m for Leu-MCA (Sigma-Aldrich) was determined from the kinetic experiments to be 38.7 μM (Ye *et al.* 2008).

Glucose handling after an oral glucose challenge

The effect of IRAP inhibitor HFI-419 on whole animal glucose handling was evaluated after an oral glucose tolerance test (OGTT) in male Sprague–Dawley rats. Twelve rats were fasted overnight (approximately 16h) prior to intravenous administration of IRAP inhibitor HFI-419 (0.5mM at a volume of 0.2mL) or vehicle (10% DMSO, 30% HPBCD, 0.2mL). Ten minutes after IV treatment, all rats were administered an oral glucose load of 300mg/100g body weight (10g/30mL) using a 13g gavage tube. Blood samples were taken from the tail vein, and glucose concentration was determined using an AccuChek meter and Advantage II glucose test strips (Roche Diagnostics). Blood was sampled immediately prior to IV treatment (basal) and at 30, 60, 120 and 240min after glucose load.

Glucose handling in streptozotocin-induced diabetic rats

To induce diabetes, 16 male Sprague–Dawley rats were fasted overnight (approximately 16h) prior to IV administration of 50mg/kg streptozotocin (STZ) dissolved in 50mM sodium citrate buffer, pH 4.5. One week after the STZ treatment, rats displaying diabetic symptoms of polyuria and hyperglycemia (elevated plasma levels of glucose of ~20mM) were randomly assigned to one of the following four treatment groups with 4 rats per group: (i) IM water (0.01mL)+IV vehicle (10% DMSO, 30% HPBCD, 200µL), (ii) IM insulin, (1IU, 0.01mL)+IV vehicle (200µL), (iii) IM water (0.01mL)+IV 5mM HFI-419 dissolved in 10% DMSO, 30% HPBCD (0.2mL) and (iv) IM insulin (1IU, 0.01mL)+IV 5mM HFI-419 (0.2mL). Blood samples were taken from the tail for determination of glucose concentration using an AccuChek meter and Advantage II glucose test strips. Blood was sampled at 0min (prior to IM and IV treatments) and at 30, 60, 120 and 240min after IV vehicle or drug treatment.

Results

L6 GLUT4myc cells were demonstrated to express IRAP by Western blot analysis in agreement with previous literature (Fig. 1A). Consistent with other insulin-responsive cells, IRAP is found in GLUT4 vesicles in L6 cells and translocates with GLUT4 to the plasma membrane after insulin stimulation (Urso *et al.* 1999, Li *et al.* 2016). Under basal conditions, a percentage reduced amount of IRAP is present at the plasma membrane via recycling endosomes (Urso *et al.* 1999, Li *et al.* 2016). L6GLUT4myc myotubes

Figure 1

Expression of IRAP and GLUT4 in differentiated (myoblasts) L6 and L6GLUT4myc cells and inhibition of IRAP by HFI419. (A) Western blot analysis of IRAP and GLUT4. SDS protein samples were prepared from 6-well plates of each cell line 8–10 days after the addition of differentiation media. Thirty micrograms was loaded in each lane. (B) Kinetic analysis of IRAP inhibition. Shown are double-reciprocal (Lineweaver–Burk) plots of IRAP activity in the absence and presence of three concentrations of HFI419. Each point represents the mean $\pm$ s.e.m. of three experiments performed in triplicate.

were stimulated with two different doses of insulin (low dose: 25 nM and high dose: 100 nM) and tested with two doses of the HFI-419 IRAP inhibitor (10^{-6} M or 10^{-7} M). HFI-419 is a competitive inhibitor of IRAP as demonstrated in the Lineweaver–Burk plot (Fig. 1B) with a K_i of 0.48 µM (Albiston *et al.* 2008). HFI-419 is highly specific for IRAP with >1000-fold lower or no affinity for related and unrelated enzymes (Albiston *et al.* 2008). In addition, a ‘BioPrint Profile’ screen by CEREP (France) against 106 known targets including GPCRs, ion channels, kinases and phosphatases demonstrated no cross-reactivity at micromolar concentrations. The IRAP inhibitor was administered for 30 min, concurrent with the insulin stimulus (Fig. 2A) or incubated for 24 h prior to insulin stimulation (Fig. 2B). A significant increase in the glucose uptake by L6GLUT4myc cells was observed with insulin stimulation at both doses used (Fig. 2). However, the incubation of cells with HFI419 for either 30 min or 24 h did not alter glucose uptake in the basal state or under insulin-stimulated conditions (Fig. 2).

Figure 2

Inhibition of IRAP does not increase glucose uptake by L6GLUT4myc muscle cells. ^3H -2DG uptake in differentiated L6GLUT4myc muscle cells incubated for (A) 30 min or (B) 24 h with HFI-419 (0, 10^{-6} M and 10^{-7} M) plus or minus insulin (0, 25 nM and 100 nM insulin). Glucose uptake was

Figure 3

IRAP inhibition does not alter glucose handling in normal rats. Adult male Sprague–Dawley rats were fasted overnight and treated with (●) HFI-419 (0.5 mM, $n=6$) or (○) vehicle ($n=6$) and an oral glucose tolerance test (OGTT) was performed as described in 'Materials and methods' section. No significant effect of HFI-419 treatment on OGTT results was observed. Data were analysed by repeated-measures 2-way ANOVA followed by Sidak's multiple comparisons test and is shown as mean $\pm$ S.E.M.

The effect of IRAP inhibition on whole body glucose handling in normal rats was investigated. Treatment with HFI-419 did not alter the response to an oral glucose challenge. No difference was observed in peak blood glucose concentrations, recorded at 30 min after glucose load. The clearance of the glucose load, as measured by the return of blood glucose concentrations to basal levels over the following hours, was not different between HFI-419 and vehicle-treated rats (Fig. 3).

In addition, the effect of IRAP inhibition on glucose handling in the STZ experimental rat model of diabetes was examined. Administration of STZ induced a diabetic state within one week with all rats displaying a basal blood glucose concentration of >23 mmol/L, which was not significantly different between the animals. STZ rats were then treated with HFI-419 with or without insulin. Treatment with insulin significantly decreased blood glucose concentrations below those of control (water)-treated rats. However, treatment with IRAP inhibitor HFI-419 did not alter the insulin response or basal glucose levels in STZ rats (Fig. 4).

calculated by normalizing the tritium content in stimulated state to that in basal state of the cells. Incubation with HFI-419 for 30 min or 24 h did not alter glucose uptake in either the basal or insulin stimulated conditions. Two-way ANOVA followed by a Tukey's *post hoc* test demonstrated that the cells responded significantly to insulin ((A) $n=4$, (B) $n=7$; $*P \leq 0.05$).

Figure 4

IRAP inhibition does not alter glucose levels in STZ-induced diabetic rats. Adult male Sprague–Dawley rats with STZ-induced diabetes were treated with HFI-419 (squares) (0.5 mM, $n=6$) or vehicle (circles) ($n=6$) plus 1 IU of insulin (filled symbols) or vehicle (open symbols). Data were analysed by 2-way ANOVA followed by Tukey's multiple comparisons test and is shown as mean $\pm$ S.E.M. No significant effect of HFI-419 treatment on glucose levels in either the basal or insulin-stimulated states was observed.

Discussion

IRAP is capable of cleaving small bioactive peptides *in vitro*; these peptide substrates include vasopressin, oxytocin, lys-bradykinin, met-enkephalin, dynorphin A (1–8), neurokinin A, neuromedin B, somatostatin and CCK-8 (Herbst *et al.* 1997, Lew *et al.* 2003b). A soluble form of IRAP is produced by the placenta during pregnancy and released into the circulation. The circulating aminopeptidase plays a role in regulating vasopressin and oxytocin levels to prevent the onset of premature labor (Tsujimoto *et al.* 1992). In addition, IRAP was demonstrated to play a role in the immune system by trimming peptides for antigen cross-presentation via major histocompatibility class I (MHC I) molecules (Saveanu *et al.* 2005). However, a direct association between the cleavage of peptide substrates by IRAP and glucose uptake in insulin-responsive cells has not been established. Although insulin stimulation results in the trafficking of IRAP to the plasma membrane, the aminopeptidase does not cleave insulin and is therefore not part of a negative feedback mechanism (Lew *et al.* 2003b). This is in contrast to the negative feedback mechanism for IRAP that has previously been proposed for oxytocin, which was based on studies where

oxytocin, an IRAP substrate, stimulated the translocation of IRAP to the plasma membrane in endothelial cells (Sano *et al.* 2005).

Utilizing the IRAP inhibitor, HFI-419, we were unable to demonstrate any changes in glucose uptake in both *in vitro* and *in vivo* systems. Using L6GLUT4myc cells, IRAP inhibition of both basal and insulin-stimulated cells did not alter glucose uptake; this was observed for both short (30-min) and 24-h incubations with HFI-419. In keeping with these results, HFI-419 did not affect peripheral glucose handling in normal rats or in the STZ-induced diabetes mellitus rats during an oral glucose tolerance test. HFI-419 had no effect on glucose handling in STZ rats with or without insulin treatment.

In contrast, we previously reported that HFI-419 potentiated glucose uptake in K^+ -evoked depolarized or cAMP-stimulated neurons (Fernando *et al.* 2008). The enhanced glucose uptake was demonstrated to be mediated by GLUT4 as the non-selective GLUT4 inhibitor, indinavir, blocked the effect. It was proposed that this effect may be due to increasing the half-life of IRAP and GLUT4 at the plasma membrane. This contrasting observation may be due to differences in GSVs in neurons compared to classic insulin-responsive cells such as muscle and adipocytes. In conclusion, our current study demonstrates that inhibition of IRAP catalytic activity does not modulate glucose uptake in insulin-responsive cells.

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

A L Albiston wrote the manuscript, designed and interpreted the *in vitro* experiments and participated in the design and interpretation of the *in vivo* experiments. M Cacador conducted the *in vitro* glucose experiments. P Sinnyah participated in the design and interpretation of the glucose *in vitro* experiments. P Burns conducted the *in vivo* glucose handling experiments and S Y Chai is the head of the laboratory who oversaw all aspects of the project from the design of the experiment to the interpretation of the results.

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