Regulation of insulin signal transduction pathway by a small-molecule insulin receptor activator

Victor D. H. DING^{*1}, Sajjad A. QURESHI^{*}, Deborah SZALKOWSKI^{*}, Zhihua LI^{*}, Dawn E. BIAZZO-ASHNAULT^{*}, Dan XIE^{*}, Kun LIU[†], A. Brian JONES[†], David E. MOLLER^{*} and Bei B. ZHANG^{*}

^{*}Department of Molecular Endocrinology, Merck Research Laboratories, PO Box 2000, 126 East Lincoln Avenue, Rahway, NJ 07065, U.S.A.,

and [†]Department of Medicinal Chemistry, Merck Research Laboratories, PO Box 2000, 126 East Lincoln Avenue, Rahway, NJ 07065, U.S.A.

Insulin regulates cellular metabolism and growth through activation of insulin receptors (IRs). We recently identified a non-peptide small-molecule IR activator (compound 2), which induced human IR tyrosine kinase activity in Chinese-hamster ovary cells expressing human IR [Qureshi, Ding, Li, Szalkowski, Biazzo-Ashnault, Xie, Saperstein, Brady, Huskey, Shen et al. (2000) *J. Biol. Chem.* **275**, 36590–36595]. Oral treatment with this compound resulted in correction of hyperglycaemia, hypertriacylglycerolaemia and hyperinsulinaemia in several rodent models of diabetes. In the present study, we have found that this compound increased tyrosine phosphorylation of the IR β -subunit and IR substrate 1 in primary rat adipocytes as well as induced phosphorylation of Akt, the 70 kDa ribosomal protein S6 kinase and glycogen synthase-3 (deactivation) in Chinese-hamster ovary cells expressing human IR. Similar to insulin, compound 2 stimulated glucose uptake, glycogen synthesis and inhibited isoprenaline-stimulated lipolysis in adipocytes. A struc-

turally related analogue (compound 3) was devoid of the above activities suggesting that the activity of compound 2 is specifically mediated by targeted IR activation. The effects of compound 2 on stimulation of glucose uptake, glycogen synthesis and inhibition of lipolysis were blocked by wortmannin, consistent with the involvement of a phosphoinositide 3-kinase-dependent pathway. In addition, compound 2, but not compound 3, exhibited additive or synergistic effects with sub-maximal concentrations of insulin in rat adipocytes. Thus the IR activator was capable of activating insulin-mediated signalling and metabolic pathways in primary adipocytes. These results demonstrate that IR activators have implications for the future development of new therapeutic approaches to Type I and Type II diabetes.

Key words: adipocytes, Akt, anti-lipolysis, insulin signal transduction, isoprenaline.

INTRODUCTION

The insulin receptor (IR) is expressed in almost all mammalian tissues, with the highest concentration found in target tissues of insulin, muscle, adipose tissue and liver [1]. The IR is an $\alpha 2/\beta 2$ tetramer. The α -subunits are located external to the plasma membrane and contain the insulin-binding sites, whereas the β -subunits are transmembrane proteins containing the tyrosine kinase domains [2,3]. Insulin binding to the IR leads to autophosphorylation and activation of the tyrosine kinase, resulting in subsequent phosphorylation of several intracellular substrates [4]. IR substrate (IRS) 1 was the first docking protein identified in the IRS family [5]. Tyrosine-phosphorylated IRS-1 protein transduces downstream signals by binding directly to the Src homology 2 ('SH2') domains of various signalling proteins, including phosphoinositide 3-kinase (PI 3-kinase). Activation of PI 3-kinase is one of the earliest steps in the insulin signalling pathway [6] and plays a major role in many insulin-regulated responses, including stimulation of glucose uptake [7–11], glycogen synthesis [12,13], anti-lipolysis [14–16], protein synthesis [17] and gene expression [18,19].

Insulin is an essential hormone for maintaining whole-body glucose homeostasis. In normal individuals, the response to increased plasma glucose levels is to increase the secretion of insulin from the β -cells of the pancreatic islets. Insulin stimulates glucose transport into peripheral tissues. In Type II diabetes, a decrease in the ability of insulin to stimulate glucose disposal, inhibit hepatic glucose production and a relative deficiency in insulin secretion lead to a complex metabolic disorder [20]. The

molecular basis for insulin resistance in Type II diabetes is complex and remains poorly understood. Several studies [21–24] have described insulin resistance manifested as deficiencies in IR signalling, such as decreases in IRS-1 and IRS-2 phosphorylation, PI 3-kinase activation and IR tyrosine kinase (IRTK) activity in human subjects and rodents with Type II diabetes. Studies using tissue-selective IR knockout mice demonstrated further the important role of insulin signalling in maintaining insulin sensitivity and glucose homeostasis [25].

In a previous study [26], we identified a small-molecule IR activator (compound 2) with insulin-mimetic and insulin-sensitizing activities in cells and animals. The aim of the present study is to characterize the effect of the compound on insulin signalling pathways involved in the stimulation of glucose uptake, anti-lipolysis and glycogen synthesis in primary adipocytes and Chinese-hamster ovary cells expressing human IR (CHO-IR). The results of these studies demonstrated that the IR activator compound is an effective insulin mimetic agent in the cellular model of insulin-sensitive adipose tissue.

MATERIALS AND METHODS

Materials

D-[U-¹⁴C]Glucose was purchased from NEN Life Science Products (Boston, MA, U.S.A.). Collagenase type 1 (359 units/mg) was obtained from Worthington Biochemical (Freehold, NJ, U.S.A.) and wortmannin was purchased from Biomol

Abbreviations used: CHO-IR, Chinese-hamster ovary cells expressing human IR; GSK-3, glycogen synthase kinase-3; IR, insulin receptor; IRS, IR substrate; IRTK, IR tyrosine kinase; p70 S6 kinase, 70 kDa ribosomal protein S6 kinase; PI 3-kinase, phosphoinositide 3-kinase.

¹ To whom correspondence should be addressed (e-mail victor_ding@merck.com).

Figure 1 Activation of IR signalling in rat adipocytes

Adipocytes were treated with insulin (In), compound 2 (Cpd 2) or compound 3 (Cpd 3) for 30 min, and the cell lysates were subjected to Western-blot analysis. Upper panel: cell lysates were separated by SDS/PAGE on a 4–20% (w/v) gel, and immunoblot analysis was performed with the monoclonal anti-phosphotyrosine antibody. The bands corresponding to the β -subunit of IR (IR- β) and IRS-1 are indicated. Lower panel: cell lysates were separated by SDS/PAGE on a 10% (w/v) gel, and immunoblotted with anti-[phospho-Akt (Ser⁴⁷³)]-specific antibody (see the Materials and methods section). The band corresponding to phosphorylated Akt (phospho-Akt) is indicated. Molecular-mass markers (in kDa) are shown on the right.

(Plymouth Meeting, PA, U.S.A.). Dinonylphthalate was obtained from ICN (Costa Mesa, CA, U.S.A.). Bovine insulin, cytochalasin B and other chemicals were purchased from Sigma (St. Louis, MO, U.S.A.). SDS/PAGE gels were purchased from Novex (San Diego, CA, U.S.A.). Compound 2 {2,5-dihydroxy-3-(1-methyl-1H-indol-3-yl)-6-phenyl-[1,4]benzoquinone} and compound 3 {2,5-dihydroxy-3-(4-methoxyphenyl)-6-phenyl-[1,4]benzoquinone} were synthesized at Merck (Rahway, NJ, U.S.A.) as described previously [27]. Stock solutions were prepared in DMSO and diluted in serum-free media containing 0.02% ascorbic acid.

Animals

Male Wistar rats were from Charles River (Wilmington, MA, U.S.A.). Epididymal adipose tissues were removed from rats weighing 120–200 g. Rats were given a standard laboratory diet. All procedures for the humane handling, care and treatment of research animals were done according to humane animal use procedures approved by the Merck Institutional Animal Care and Use Committee.

Cell culture and treatment

CHO.IR cells (a gift from Dr Richard Roth, Stanford University, Stanford, CA, U.S.A.) were cultured and maintained as described previously [28]. Cells were starved in serum-free media for 2 h before treatment with insulin, compound or vehicle.

Figure 2 Phosphorylation of p70 S6 kinase in CHO.IR cells

Cells were treated for 30 min in the absence (control) or presence of insulin (In), compound 2 (Cpd 2) or compound 3 (Cpd 3). In a subset of samples, cells were preincubated with 200 nM wortmannin (Wort.) for 20 min before treatment with insulin or compound 2. Cell lysates were prepared and proteins were separated by SDS/PAGE (4–20% gel). (A) Phosphorylation of p70 S6 kinase. Immunoblot of phosphorylation of p70 S6 kinase (phos-p70 S6 kinase) was detected with the anti-(phospho-p70 S6 kinase) monoclonal antibody. (B) Total p70 S6 kinase protein. Immunoblot of total p70 S6 kinase was detected with the anti-(p70 S6 kinase) monoclonal antibody. Molecular-mass markers (in kDa) are shown on the right.

Measurement of anti-lipolysis in adipocytes by glycerol release

Isolated adipocytes were obtained from rat epididymal adipose tissue as described previously [14]. The adipocytes (200 μ l of packed cells) were incubated at 37 °C in a total 1 ml of Krebs–Ringer bicarbonate buffer [120 mM NaCl, 4.7 mM KCl, 1 mM CaCl₂ · 2H₂O, 1.2 mM KH₂PO₄, 2.5 mM MgSO₄, 33.7 mM NaHCO₃ (pH 7.4)] containing 1% BSA (essentially fatty-acid-free). Cells were incubated with insulin, compound or vehicle in the presence of 0.1 μ M isoprenaline for 20 min. After incubation, 300 μ l of the reaction mixture was immediately removed and centrifuged on top of 200 μ l of dinonylphthalate for 1 min at 325 g in an Eppendorf centrifuge to separate the medium from cells. The amount of glycerol released in 200 μ l of medium was measured with the Sigma 337-A kit. Anti-lipolysis was expressed as the percentage of maximal lipolysis of adipocytes stimulated with isoprenaline (0.1 μ M).

Measurement of glucose uptake

Glucose uptake in adipocytes was performed as described previously [29] with minor modifications. Adipocytes were stimulated with insulin, compound or vehicle for 30 min and then incubated for another 30 min at 37 °C in the presence of D-[U-¹⁴C]glucose (0.1 μ Ci/ μ mol). Cytochalasin B was added at the same time to one of the treatments as a negative control. The effect of wortmannin (200 nM) was assessed by incubating the adipocytes with the inhibitor for 20 min before the addition of insulin or compound. Glucose uptake was measured by the incorporation of [¹⁴C]glucose into cells.

Measurement of glycogen synthesis

Glycogen synthesis was determined from the incorporation of [¹⁴C]glucose into glycogen [30,31], with modifications. The reaction mixture (500 μ l) containing 200 μ l of packed adipocytes, 0.5% glycogen, and D-[U-¹⁴C]glucose (0.2 μ Ci/ μ mol final specific radioactivity) in Krebs–Ringer bicarbonate buffer was incubated for 90 min at 37 °C in O₂/CO₂ (19:1). After incubation, 100 μ l of 1 M NaOH was added and 500 μ l was spotted on to 2 cm × 8 cm filters (3MM; Whatman, Maidstone, Kent, U.K.). Filters were washed in 66% (v/v) ethanol three times for 10 min each at

Figure 3 Phosphorylation of GSK-3 in CHO.IR cells

Cells were treated for 30 min in the absence (control) or presence of insulin (In), compound 2 (Cpd 2) or compound 3 (Cpd 3). In a subset of samples, cells were preincubated with 200 nM wortmannin (Wort.) for 20 min before treatment with insulin or compound 2. Cell lysates were prepared and proteins were separated by SDS/PAGE (4–20% gel). (A) Phosphorylation of GSK-3. Immunoblot of phosphorylated GSK-3 (phos-GSK-3) was detected with the anti-(phospho-GSK-3 α/β) antibody. (B) Total GSK-3 protein. Immunoblot of total GSK-3 proteins were detected with anti-(GSK-3) monoclonal antibody. Molecular-mass markers (in kDa) are shown on the right.

25 °C, immersed in acetone, dried and the radioactivity incorporated into glycogen was counted.

SDS/PAGE and Western-blot analysis

Phosphorylation of IRS-1, Akt, glycogen synthase kinase-3 (GSK-3) and 70 kDa ribosomal protein S6 kinase (p70 S6 kinase) was detected by Western blotting. Cell lysates of adipocytes or CHO.IR cells were fractionated by SDS/PAGE on 4–12% (w/v) or 4–20% (w/v) gels. The proteins were transferred on to nitrocellulose filters, which were subsequently incubated with antibodies directed against phosphotyrosine, phosphorylated Akt (Ser⁴⁷³), phosphorylated GSK-3 α/β (Ser^{21/9}) or phosphorylated p70 S6 kinase (New England Biolabs, Beverly, MA, U.S.A.). An anti-(GSK-3) monoclonal antibody (Upstate Biotechnology, Lake Placid, NY, U.S.A.) and an anti-(p70 S6 kinase) polyclonal antibody (New England Biolabs) were used to determine the levels of total GSK-3 and p70 S6 kinase respectively. After incubation with horseradish peroxidase-conjugated second antibodies, the signals were detected with the Renaissance enhanced chemiluminescence kit (NEN Life Science Products).

Other methods

Protein concentrations were determined using the Bradford protein assay reagent (Bio-Rad Laboratories, Hercules, CA, U.S.A.) following the manufacturer's instructions. Significant differences between treatment and control groups were assessed using Student's *t* test. Values are reported as means $\pm$ S.E.M.

RESULTS

Activation of IR signal transduction in adipocytes and CHO.IR cells

In a previous study [26], we have reported that compound 2 activated human IRTK activity with an EC₅₀ of 300 nM. In contrast, compound 3 was not effective in activating IRTK in CHO.IR cells. Compound 2, but not compound 3, stimulated

Figure 4 Insulin and compound 2 stimulate glucose uptake in adipocytes

Adipocytes were incubated for 30 min with insulin (In), compound 2 (Cpd 2) or compound 3 (Cpd 3) alone, or compound 2 plus insulin (Cpd 2 + In). Cells were incubated for a further 30 min in the presence of D-[U-¹⁴C]glucose to measure glucose uptake. In a subset of samples, cells were preincubated with 200 nM wortmannin (Wort.) for 20 min before treatment with insulin or compound 2. ***P* < 0.01 compared with basal; **P* < 0.05 compared with insulin (0.1 nM) or compound 2 (1 μM). Results are means $\pm$ S.E.M. of six determinations derived from two independent experiments.

tyrosine phosphorylation of the β -subunit of IR and IRS-1 as well as phosphorylation of Akt in CHO.IR cells.

In the present study, we determined whether compound 2 was able to stimulate tyrosine phosphorylation of the IR and IRS-1 in primary rat adipocytes, which express physiological levels of IR. Rat adipocytes were treated with insulin or test compounds and then lysed. Lysates were subjected to Western-blot analysis using antibodies against phosphotyrosine or Akt phosphorylated at Ser⁴⁷³. As shown in Figure 1, insulin and compound 2 stimulated tyrosine phosphorylation of the IR β -subunit and IRS-1 as well as phosphorylation of Akt in a dose-dependent manner. Compound 3 did not stimulate phosphorylation of the IR β -subunit, IRS-1 or Akt in adipocytes. These results confirmed that compound 2 is specific in activating proximal steps in the insulin signal transduction pathway.

p70 S6 kinase is another key enzyme downstream of the PI 3-kinase pathway. Activation of p70 S6 kinase by insulin was accompanied by an increase in phosphorylation of serine/threonine sites. Compound 2, but not compound 3, and insulin stimulated phosphorylation of p70 S6-kinase in CHO.IR cells (Figure 2A). Although activation of p70 S6 kinase by insulin is PI 3-kinase-dependent, PI 3-kinase-independent processes of p70 S6 kinase activation have been reported [4]. Our findings in Figure 2(A) indicated that the phosphorylation of p70 S6 kinase by insulin and compound 2 was completely inhibited by wortmannin. Total p70 S6 kinase protein level of each sample was constant (Figure 2B).

We also carried out experiments to determine whether compound 2 activates downstream steps in the insulin signal transduction pathway that are involved in glycogen synthesis. GSK-3 is involved in regulation of glycogen synthase activity and is phosphorylated and inactivated by Akt in response to insulin stimulation [32,33]. Lysates from CHO.IR cells treated with insulin or test compounds were subjected to Western-blot analysis using antibodies against GSK-3. Similar to insulin, compound

Figure 5 Inhibition of isoprenaline-stimulated lipolysis by insulin and compound 2

Cells were incubated for 20 min with insulin (In), compound 2 (Cpd 2) or compound 3 (Cpd 3), or compound 2 plus insulin (Cpd 2 + In) in the presence of 0.1 μM isoprenaline (ISO). Lipolysis of adipocytes was determined by measuring glycerol released from cells. Maximum (100%) lipolysis was defined in cells treated with isoprenaline only. * $P < 0.05$ compared with isoprenaline treatment alone. Results are means $\pm$ S.E.M. of six determinations derived from two independent experiments.

2 stimulated phosphorylation of both the α and β forms of GSK-3 in a dose-dependent manner (Figure 3A). Maximal GSK-3 phosphorylation or inactivation in response to insulin and compound 2 occurred at 5 min (results not shown). Phosphorylation of GSK-3 by insulin and compound 2 was blocked by preincubation of the cells with wortmannin, suggesting the involvement of a PI 3-kinase-dependent pathway [6]. In contrast, compound 3 did not stimulate phosphorylation of GSK-3. The level of total GSK-3 protein in the lysates remained unchanged (Figure 3B).

Effect of compounds on glucose uptake

Glucose transport across the cell plasma membrane is the first step of glucose metabolism in the target tissue. As shown in Figure 4, 10 nM insulin maximally stimulated glucose uptake in adipocytes. Compound 2 alone significantly stimulated glucose uptake at 10 μM, but not at 1 μM. However, a combination of 1 μM compound 2 with the sub-maximal effective concentration of insulin (0.1 nM) resulted in significant stimulation of glucose uptake, suggesting that compound 2 exerted both insulin-mimetic and insulin-sensitizing activities in adipocytes. The effect of insulin and compound 2 on stimulation of glucose uptake was eliminated by preincubation of adipocytes with wortmannin. Compound 3 had no effect on glucose uptake at 10 μM.

Inhibition of isoprenaline-stimulated lipolysis in adipocytes

Since the endogenous level of lipolysis is low in adipocytes, we used isoprenaline-stimulated (0.1 μM) lipolysis to verify the anti-lipolytic activity of insulin or compounds. Insulin (10 nM) or compound 2 (10 μM) alone significantly inhibited lipolysis (Figure 5). An additive effect was observed at low concentrations of insulin (0.1 nM) plus compound 2 (1 μM), whereas compound

Figure 6 Effect of wortmannin on isoprenaline-stimulated lipolysis in adipocytes

Cells were preincubated in the absence or presence of wortmannin (Wort.) for 20 min before addition of insulin (In), compound 2 (Cpd 2) or a combination of insulin and compound 2 (In + Cpd 2). All samples were stimulated with 0.1 μM isoprenaline (ISO). ** $P < 0.01$ compared with compound 2 (3 μM). Results are means $\pm$ S.E.M. of six determinations derived from two independent experiments.

3 showed no effect at 30 μM. Pretreatment with wortmannin completely reversed the inhibition of lipolysis induced by insulin or insulin plus compound 2 (Figure 6), indicating the involvement of a PI 3-kinase-dependent pathway. In the present study, insulin was able to inhibit approx. 60–80% of the lipolysis mediated by cAMP following isoprenaline stimulation. Earlier studies [15,34] have shown that the anti-lipolytic effect of insulin was most pronounced at low intracellular concentrations of cAMP. Incomplete inhibition of lipolysis by insulin was observed in cells, possibly due to high cAMP levels [34,35].

Stimulation of glycogen synthesis

Glycogen accumulation in cells is dependent on the dose of glucose and is regulated by glycogen synthase, the rate-limiting enzyme in glycogen synthesis. To avoid the increasing number of lysed adipocytes during the incubation period, a minimal incubation time of 90 min was used to compare the incorporation of [14 C]glucose into glycogen. [14 C]Glucose incorporation into glycogen was significantly increased in the presence of 10 nM insulin (Figure 7). Compound 2 (10 μM) significantly stimulated glucose incorporation when compared with the basal level. Although compound 2 alone at 1 μM did not affect glycogen synthesis, it significantly stimulated glycogen synthesis ($P < 0.01$) in the presence of 0.1 nM insulin. Compound 3 was again ineffective at 30 μM. The incorporation of [14 C]glucose into glycogen stimulated by insulin or compound 2 was completely inhibited by wortmannin (Figure 8).

DISCUSSION

Type II diabetes is a complex metabolic disorder characterized by abnormal insulin secretion caused by impaired β -cell function and insulin resistance in target tissues [36]. Indeed, defects

Figure 7 Insulin and compound 2 stimulate glycogen synthesis in adipocytes

Cells were incubated for 90 min with insulin (In), compound 2 (Cpd 2) or compound 3 (Cpd 3), or compound 2 plus insulin (Cpd 2 + In) in the presence of D-[U- 14 C]glucose. Glycogen synthesis was expressed in pmol of [14 C]glucose incorporated into glycogen/ml of packed cells. ** $P < 0.01$ between insulin (10 nM) and basal, and between compound 2 (1 µM) plus insulin (0.1 nM) and compound 2 (1 µM) alone; * $P < 0.05$ compared with basal. Results are means $\pm$ S.E.M. of six determinations derived from two independent experiments.

in IR and its signal transduction pathway have been found in insulin-resistant patients [37], including decreased IR and IRS-1 phosphorylation and PI 3-kinase activity [38]. Therefore augmenting IR signalling or stimulating IR activation is a potential approach to improving insulin sensitivity in Type II diabetes. Previously [26], we demonstrated that small-molecule IR activators were capable of stimulating IR and Akt phosphorylation in CHO. IR cells. The results of the present study illustrated that the activator compound was also able to mimic the effect of insulin on GSK-3 inactivation and p70 S6 kinase activation, two downstream steps in the insulin signalling pathway that are important for the glucose metabolism and glycogen synthesis.

In the present study, we characterized the effects of compound 2 on proximal steps of insulin signal transduction pathway in adipocytes, a cellular model for an insulin target tissue. The advantage of using primary adipocytes is that these cells express physiological levels of the IR and downstream signalling molecules compared with transfected cell lines, which overexpress one or multiple components of the insulin signalling pathway. Adipocytes also provide a unique system for investigating metabolic effects of insulin. Compound 2, but not the inactive analogue compound 3, stimulated phosphorylation of IR β -subunits and IRS-1 as well as signals downstream of Akt. In functional assays, compound 2 stimulated glucose uptake, inhibited isoprenaline-stimulated lipolysis and increased glycogen synthesis in adipocytes. These results indicated that compound 2 was capable of mimicking the metabolic effects of insulin in adipocytes.

Adipocytes have been reported to contain two distinct IR populations, one with normal kinase activity and the other with a defective kinase [39]. In Type II diabetes, the ratio of the two receptor populations could favour the defective receptor population. Thus activation of kinase activity may potentiate insulin signal transduction and improve insulin resistance. IR activator compound 2 was found to activate IRTK activity of β -subunits

Figure 8 Effect of wortmannin on insulin or compound 2-stimulated glycogen synthesis

Cells were preincubated with or without wortmannin (Wort.; 200 nM) for 20 min before incubation in the absence (basal) or presence of either insulin (In) or compound 2 (Cpd 2). ** $P < 0.01$ compared with basal; and * $P < 0.05$ compared with Cpd 2 (10 µM). Results are means $\pm$ S.E.M. of six determinations derived from two independent experiments.

both *in vitro* and *in vivo* [26]. Compound 2 significantly increased insulin-stimulated IRTK activity in liver of normal mice. In the present study, compound 2 at a high concentration (10 µM) exhibited insulin-mimetic activity and exerted insulin-sensitizing activity at a low concentration (1 µM). Treatment with both insulin and compound 2 at sub-efficacious doses resulted in a significant increase in glucose uptake, glycogen synthesis and anti-lipolysis in adipocytes, indicating that both insulin and compound 2 activate the same signal transduction pathway. The ability of compound 2 to exert an insulin-mimetic and insulin-sensitizing effect in adipocytes may explain the fact that compounds of this class are capable of improving insulin sensitivity and lowering glucose in animal models with hyperglycaemia and hyperinsulinaemia [26,28].

There is an apparent discrepancy in the effectiveness of compound 2 in stimulating glucose uptake and glycogen synthesis and inhibition of lipolysis. Since these downstream steps in the insulin signalling pathway involve activation/inhibition of complex networks of adapter proteins, kinases, phosphatases and other effector molecules, it is possible that compound 2 has differential effects on these signalling steps. Further experiments will be necessary to fully address this issue.

In the insulin signalling pathway, activation of PI 3-kinase is one of the earliest steps and plays a major role in many insulin-regulated responses. Several kinases appear to carry the signal initiated by PI 3-kinase activation to its functional destination, including the serine/threonine kinase Akt. The involvement of Akt in GLUT 4 translocation and glucose uptake has been reported in different cell types [40–43]. PI 3-kinase and Akt are two important signals in the regulation of glucose homeostasis in the target cells. The results in the present study demonstrate that the cellular effects of both insulin and compound 2 were blocked by the PI 3-kinase inhibitor wortmannin, indicating the involvement of a common PI 3-kinase-dependent pathway.

Taken together, the results of the present study illustrate that the small-molecule IR activator compound 2 exerts both insulin-mimetic and -sensitizing effects in CHO. IR cells and adipocytes. These agents may provide useful tools to dissect further the signal transduction pathways downstream of the IR.

REFERENCES

- 1 Cheatham, B. and Kahn, C. R. (1995) Insulin action and the insulin signaling network. *Endocrin. Rev.* **16**, 117–142
- 2 Van Obberghoe, E., Kasuga, M., LeCam, A., Hedo, J. A. and Harison, L. C. (1981) Biosynthetic labeling of insulin receptor: studies of subunits in cultured human IM-9 lymphocytes. *Proc. Natl. Acad. Sci. U.S.A.* **78**, 1052–1056
- 3 Kasuga, M., Fujita-Yamaguchi, Y., Blithe, D. and Kahn, C. R. (1983) Tyrosine-specific protein kinase activity is associated with the purified insulin receptor. *Proc. Natl. Acad. Sci. U.S.A.* **80**, 2137–2141
- 4 Taha, C. and Klip, A. (1999) The insulin signaling pathway. *J. Membr. Biol.* **169**, 1–12
- 5 White, M. F., Maron, R. and Kahn, C. R. (1985) Insulin rapidly stimulates tyrosine phosphorylation of a Mr-185,000 protein in intact cells. *Nature (London)* **318**, 183–186
- 6 Shepherd, P. R., Withers, D. J. and Siddle, K. (1998) Phosphoinositide 3-kinase: the key switch mechanism in insulin signaling. *Biochem. J.* **333**, 471–490
- 7 Tsakiridis, T., McDowell, H. E., Walker, T., Downes, C. P., Hundal, H. S., Vraic, M. and Klip, A. (1995) Multiple roles of phosphatidylinositol 3-kinase in regulation of glucose transport, amino acid transport, and glucose transporters in L-6 skeletal muscle cells. *Endocrinology* **136**, 4315–4322
- 8 Hara, K., Yonezawa, K., Sakaue, H., Ando, A., Kotani, K., Kitamura, T., Kitamura, Y., Ueda, H., Stephens, L., Jackson, T. R. et al. (1994) 1-Phosphatidylinositol 3-kinase activity is required for insulin-stimulated glucose transport but not for RAS activation in CHO cells. *Proc. Natl. Acad. Sci. U.S.A.* **91**, 7415–7419
- 9 Cheatham, B., Vlahos, C. J., Cheatham, L., Wang, L., Blenis, J. and Kahn, C. R. (1994) Phosphatidylinositol 3-kinase activation is required for insulin stimulation of pp70 S6 kinase, DNA synthesis, and glucose transporter translocation. *Mol. Cell. Biol.* **14**, 4902–4911
- 10 Yeh, J., Gulve, E. A., Rameh, L. and Birnbaum, M. J. (1995) The effects of wortmannin on rat skeletal muscle. *J. Biol. Chem.* **270**, 2107–2111
- 11 Le Marchand-Brustel, Y., Gautier, N., Cormont, M. and Van Obberghen, E. (1995) Wortmannin inhibits the action of insulin but not that of okadaic acid in skeletal muscle: comparison with fat cells. *Endocrinology* **136**, 3564–3570
- 12 Shepherd, P. R., Nave, B. T. and Siddle, K. (1995) Insulin stimulation of glycogen synthesis and glycogen synthase activity is blocked by wortmannin and rapamycin in 3T3-L1 adipocytes: evidence for the involvement of phosphoinositide 3-kinase and p70 ribosomal protein-S6 kinase. *Biochem. J.* **305**, 25–28
- 13 Carlsen, J., Christiansen, K. and Vinten, J. (1997) Insulin stimulated glycogen synthesis in isolated rat hepatocytes: effect of protein kinase inhibitors. *Cell. Signalling* **9**, 447–450
- 14 Okada, T., Kawano, Y., Sakakibara, T., Hazeki, O. and Ui, M. (1994) Essential role of phosphatidylinositol 3-kinase in insulin-induced glucose transport and antilipolysis in rat adipocytes. *J. Biol. Chem.* **269**, 3568–3573
- 15 Li, J., Elberg, G., Sekar, N., He, Z. B. and Shechter, Y. (1997) Antilipolytic actions of vanadate and insulin in rat adipocytes mediated by distinctly different mechanism. *Endocrinology* **138**, 2274–2279
- 16 Morimoto, C., Tsujita, T. and Okuda, H. (1998) Antilipolytic actions of insulin on basal and hormone-induced lipolysis in rat adipocytes. *J. Lipid Res.* **39**, 957–962
- 17 Mendez, R., Myers, Jr, M., White, M. F. and Rhoads, R. E. (1996) Stimulation of protein synthesis, eukaryotic translation initiation factor 4E phosphorylation, and PHAS-I phosphorylation by insulin requires insulin receptor substrate 1 and phosphatidylinositol 3-kinase. *Mol. Cell. Biol.* **16**, 2857–2864
- 18 Sutherland, C., Waltner-Law, M., Gnudi, L., Kahn, B. B. and Granner, D. K. (1998) Activation of the ras mitogen-activated protein kinase-ribosomal protein kinase pathway is not required for the repression of phosphoenolpyruvate carboxykinase gene transcription by insulin. *J. Biol. Chem.* **273**, 3198–3204
- 19 Valverde, A. M., Navarro, P., Tervel, T., Conejo, R., Benito, M. and Lorenzo, M. (1999) Insulin and insulin-like growth factor 1 up-regulate GLUT 4 gene expression in fetal brown adipocytes, in a phosphoinositide 3-kinase-dependent manner. *Biochem. J.* **337**, 397–405
- 20 Kahn, C. R. (1994) Banting Lecture. Insulin action, diabetogenes, and the cause of type II diabetes. *Diabetes* **43**, 1066–1084
- 21 Goodyear, L. J., Giorgino, F., Sherman, L. A., Carey, J., Smith, R. J. and Dohm, G. L. (1995) Insulin receptor phosphorylation, insulin receptor substrate-1 phosphorylation, and phosphatidylinositol 3-kinase activity are decreased in intact skeletal muscle strips from obese subjects. *J. Clin. Invest.* **95**, 2195–2204
- 22 Caro, J. F., Sinha, M. K., Raju, S. M., Ittoop, O., Pories, W. J., Flickinger, E. G., Meelheim, D. and Dohm, G. L. (1987) Insulin receptor kinase in human skeletal muscle from obese subjects with and without non-insulin dependent diabetes. *J. Clin. Invest.* **79**, 1330–1337
- 23 Kerouz, N. J., Horsch, D., Pons, S. and Kahn, C. R. (1997) Differential regulation of insulin receptor substrate-1 and -2 (IRS-1 and IRS-2) and phosphatidylinositol 3-kinase isoforms in liver and muscle of the obese diabetic (ob/ob) mouse. *J. Clin. Invest.* **100**, 3164–3172
- 24 Folli, F., Saad, M. J. A., Backer, J. M. and Kahn, C. R. (1993) Regulation of phosphatidylinositol 3-kinase activity in liver and muscle of animal models of insulin-resistant and insulin-deficient diabetes mellitus. *J. Clin. Invest.* **92**, 1787–1794
- 25 Michael, M. D., Kulkarni, R. N., Postic, C., Previs, S. F., Shulman, G. I., Magnuson, M. A. and Kahn, C. R. (2000) Loss of insulin signaling in hepatocytes leads to severe insulin resistance and progressive hepatic dysfunction. *Mol. Cell* **6**, 87–97
- 26 Qureshi, S. A., Ding, V., Li, Z., Szalkowski, D., Biazzo-Ashnault, D. E., Xie, D., Saperstein, R., Brady, E., Huskey, S., Shen, X. et al. (2000) Activation of insulin signal transduction pathway and anti-diabetic activity of small molecule insulin receptor activators. *J. Biol. Chem.* **275**, 36590–36595
- 27 Liu, K., Xu, L., Szalkowski, D., Li, Z., Ding, V., Kwei, G., Huskey, S., Moller, D. E., Heck, J. V., Zhang, B. B. and Jones, A. B. (2000) Discovery of a potent, highly selective, and orally efficacious small-molecule activator of the insulin receptor. *J. Med. Chem.* **43**, 3487–3494
- 28 Zhang, B., Salituro, G., Szalkowski, D., Li, Z., Zhang, Y., Royo, I., Vilella, D., Diez, M. T., Pelaez, F., Ruby, C. et al. (1999) Discovery of a small molecule insulin mimetic with antidiabetic activity in mice. *Science (Washington, D.C.)* **284**, 974–977
- 29 Kahn, B. B., Simpson, I. A. and Cushman, S. W. (1988) Divergent mechanisms for the insulin resistant and hyperresponsive glucose transport in adipose cells from fasted and refed rats. *J. Clin. Invest.* **82**, 691–699
- 30 Thomas, J. A., Schlender, K. K. and Lerner, J. (1968) A rapid filter paper assay for UDP glucose-glycogen glucosyltransferase, including an improved biosynthesis of UDP ¹⁴C-glucose. *Anal. Biochem.* **25**, 486–499
- 31 Agius, L., Peak, M. and Alberti, K. G. (1990) Regulation of glycogen synthesis from glucose and gluconeogenic precursors in periportal and perivenous rat hepatocytes. *Biochem. J.* **266**, 91–102
- 32 Nikoulina, S. E., Ciaradi, T. P., Mudaliar, S., Mohideen, P., Carter, L. and Henry, R. R. (2000) Potential role of glycogen synthase kinase-3 in skeletal muscle insulin resistance of type 2 diabetes. *Diabetes* **49**, 263–271
- 33 Cross, D. A., Alessi, D. R., Cohen, P., Andjelkovich, M. and Hemmings, B. A. (1995) Inhibition of glycogen synthase kinase-3 by insulin mediated protein kinase B. *Nature (London)* **378**, 785–789
- 34 Mooney, R. A., Ebersohl, R. D. and McDonald, J. M. (1984) Insulin-mediated antilipolysis in permeabilized rat adipocytes. *J. Biol. Chem.* **259**, 7701–7704
- 35 Castan, I., Wijkander, J., Manganiello, V. and Degerman, E. (1999) Mechanisms of inhibition of lipolysis by insulin, vanadate and peroxovanadate in rat adipocytes. *Biochem. J.* **339**, 281–289
- 36 Pessin, J. E. and Saltiel, A. R. (2000) Signaling pathways in insulin action: molecular targets of insulin resistance. *J. Clin. Invest.* **106**, 165–169
- 37 Dozio, N., Micossi, P., Galimberti, G., Sartori, S., Pozza, G., Dosio, F., Savi, F., Gerundini, P. G., Fazio, F., Chiomello, G. et al. (1992) *In vivo* demonstration of insulin-receptor defect with ¹²⁵I-labeled insulin and scintigraphic scanning in severe insulin resistance. *Diabetes Care* **15**, 651–656
- 38 Goodyear, L. J., Giorgino, F., Sherman, L. A., Carey, J., Smith, R. J. and Dohm, G. L. (1995) Insulin receptor phosphorylation, insulin receptor substrate-1 phosphorylation and phosphatidylinositol 3-kinase activity are decreased in intact skeletal muscle strips for obese subjects. *J. Clin. Invest.* **95**, 2195–2204
- 39 Brillion, D. J., Freidenberg, G. R., Henry, R. R. and Olefsky, J. M. (1989) Mechanism of defective insulin-receptor kinase activity in NIDDM: evidence for two receptor populations. *Diabetes* **38**, 397–403
- 40 Cong, L. N., Chen, H., Li, Y., Zhon, L., McGibbon, M. A., Taylor, S. I. and Quon, M. J. (1997) Physiological role of Akt in insulin-stimulated translocation of GLUT 4 in transfected rat adipose cells. *Mol. Endocrinol.* **11**, 1881–1890
- 41 Smith, U., Carvalho, E., Mosialou, E., Beguinot, F., Formisano, P. and Rondinone, C. (2000) PKB inhibition prevents the stimulatory effect of insulin on glucose transport and protein translocation but not the antilipolytic effect in rat adipocytes. *Biochem. Biophys. Res. Commun.* **268**, 315–320
- 42 Hajdouch, E., Alessi, D. R., Hemmings, B. A. and Hundal, H. S. (1998) Constitutive activation of protein kinase B α by membrane targeting promotes glucose and system A amino acid transport, protein synthesis and inactivation of glycogen synthase kinase-3 in L6 muscle cells. *Diabetes* **47**, 1006–1019
- 43 Wang, Q., Somwar, R., Bilan, P. J., Liu, Z., Jin, J., Woodgett, J. R. and Klip, A. (1999) Protein kinase B/Akt participates in GLUT 4 translocation by insulin in L6 myoblasts. *Mol. Cell. Biol.* **19**, 4008–4018

Received 6 May 2002; accepted 30 May 2002

Published as BJ Immediate Publication 30 May 2002, DOI 10.1042/BJ20020708