

Inhibitors of phosphatidylinositol 3-kinase amplify insulin release from islets of lean but not obese mice

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Abstract

We examined the effects of phosphatidylinositol 3-kinase (PI3K) inhibition by wortmannin or LY294002 on glucose-induced secretion from mouse islets. Islets were collagenase isolated and perfused or subjected to Western blot analyses and probed for insulin receptor-signaling components. In agreement with previous studies, mouse islets, when compared with rat islets, were minimally responsive to 10 mM glucose stimulation. The inclusion of 50 nM wortmannin or 10 μ M LY294002 significantly amplified 10 mM glucose-induced release from mouse islets. The effect of wortmannin was abolished by the calcium channel antagonist nitrendipine or by lowering the glucose level to 3 mM. Wortmannin had no effect on 10 mM α -ketoisocaproate-induced secretion. In contrast

to its potentiating effect on islets from CD-1 mice, wortmannin had no effect on 10 mM glucose-induced release from ob/ob mouse islets. Western blot analyses revealed the presence of the insulin receptor, insulin receptor substrate proteins 1 and 2 and PI3K in CD-1 islets. These results support the concept that a PI3K-dependent signaling pathway exists in β -cells and that it may function to restrain glucose-induced insulin secretion from β -cells. They also suggest that, as insulin resistance develops in peripheral tissues, a potential result of impaired PI3K activation, the same biochemical anomaly in β -cells promotes a linked increase in insulin secretion to maintain glucose homeostasis.

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Introduction

Often overlooked in studies utilizing mouse islets is the disparity that characterizes this species islet responses to glucose when compared with rat or human islet responses (Gerich *et al.* 1974, Pagliara *et al.* 1974, Berglund 1980, O'Conner *et al.* 1980, Curry 1986, Van Haeften *et al.* 1990, Elahi 1996). For example, over 30 years ago, it was reported (Malaisse & Malaisse-Lagae 1968) that glucose-induced insulin release was reduced from freshly studied, statically incubated mouse pancreatic pieces when compared with rat responses. This difference has since been ascribed to a profound reduction in the magnitude of the second phase secretory response to the hexose from mouse islets (Lenzen 1979, Berglund 1980, Ma *et al.* 1995, Zawalich *et al.* 1995b, Seghers *et al.* 2000). More recent studies have attributed this reduction to alterations in signaling via the phospholipase C (PLC)/protein kinase C pathway (Zawalich *et al.* 1995b, Zawalich 1996, Zawalich & Zawalich 1996a). When compared with both the exuberant second phase insulin secretory response (Charles *et al.* 1973, Henquin & Lambert 1976, Zawalich *et al.* 1989b) and robust generation of inositol phosphates (IPs) in studies using rat islets (Best & Malaisse 1983, 1984, Zawalich *et al.* 1991), minimal effects are observed in

similarly treated mouse islets (Zawalich *et al.* 1995b, Zawalich 1996, Sato & Henquin 1998). Species differences in the expression of several PLC isozymes have also been reported and may account for the reduction in both IP accumulation and second phase release from mouse islets (Zawalich *et al.* 1995b).

Transgenic and knockout mice are being increasingly utilized as models to study the biochemical pathways responsible for the emergence of insulin resistance and their potential involvement in the pathogenesis of type 2 diabetes (Araki *et al.* 1994, Tamemoto *et al.* 1994, Kido *et al.* 2000). These and other studies (Harbeck *et al.* 1996) have demonstrated that elements of the insulin signaling pathways characterized in peripheral tissues such as liver, muscle and adipose cells (Virkamäki *et al.* 1999) exist in β -cells as well. Most recently, it has been reported (Kubota *et al.* 2000) that islets isolated from insulin receptor substrate-2 (IRS-2)-deficient mice exhibit augmented insulin secretory responses to glucose stimulation. These findings suggest that IRS-2 activation of phosphatidylinositol 3-kinase (PI3K) may participate in a negative feedback system that functions to regulate insulin secretion. Also consistent with the concept that insulin signaling regulates β -cell secretory responses are many previous studies demonstrating that insulin inhibits its own

secretion (Iversen & Miles 1971, Loreti *et al.* 1974, Ammon & Verspohl 1976, Liljenquist *et al.* 1978, Ammon *et al.* 1991).

We recently demonstrated (Zawalich & Zawalich 2000) that the fungal metabolite wortmannin, a specific PI3K inhibitor at nanomolar concentrations (Acaro & Wymann 1993, Okada *et al.* 1994, Yeh *et al.* 1995), amplified 8 mM glucose-induced insulin secretion from perfused rat islets. Since PI3K activation by insulin receptor substrate proteins appears to determine liver, muscle and adipose tissue sensitivity to insulin, we suggested that this signaling pathway might provide a means for the β -cell to monitor insulin sensitivity in peripheral tissues and to adjust their secretory responses accordingly. Because of the existence of pronounced species differences in glucose responses between rat and mouse islets and the almost exclusive use of mice as transgenic models of diseases, we decided to assess the impact of several structurally distinct PI3K inhibitors on glucose-induced release from perfused mouse islets. In addition, mouse islets were subjected to Western blot analyses and probed for the presence of PI3K, the insulin receptor (IR), IRS-1 and IRS-2.

Materials and Methods

Islet isolation

The detailed methodologies employed to assess insulin output from collagenase-isolated islets have been previously described (Zawalich & Zawalich 1988). Male Sprague-Dawley (SD) rats (weight at time of study 350–475 g) and male CD-1 mice (22–38 g) were purchased from Charles River (Raleigh, NC, USA). Female ob/ob mice (40–52 g, stock number 000632, former name C57BL/6J-Lep ob, current name B6.V-Lep ob) and their lean littermates (19–22 g) were purchased from Jackson Laboratories (Bar Harbor, ME, USA). All animals were treated in a manner which complied with the NIH Guidelines for the Care and Use of Laboratory Animals (NIH publication no. 85–23, revised 1985). The animals were fed *ad libitum*. After intraperitoneal pentobarbital sodium (Nembutal, 50 mg/kg; Abbott, North Chicago, IL, USA)-induced anesthesia, islets were isolated by collagenase digestion and handpicked, using a glass loop pipette, under a stereo microscope. They were free of visual exocrine contamination.

Perfusion studies

Groups of 14–18 isolated islets were loaded onto nylon filters (Tetko, Inc., Briarcliff Manor, NY, USA) and perfused in a Krebs–Ringer bicarbonate (KRB) buffer at a flow rate of 1 ml/min for 30 min, usually with 3 mM glucose, to establish basal and stable insulin secretory rates. In experiments with α -ketoisocaproate (KIC), glucose was

omitted during this time. After this 30-min stabilization period they were then perfused with the appropriate agonist or agonist combinations as indicated in the figure legends and Results section. Wortmannin or LY294002 were dissolved in dimethyl sulfoxide and comparable amounts of this diluent were added to control solutions. Perfusate solutions were gassed with 95% O₂/5% CO₂ and maintained at 37 °C. Insulin released into the medium was measured by RIA (Albano *et al.* 1972).

Western blot studies

Freshly isolated islets from CD-1 mice were suspended in a 2:1 ratio of islets per μ l of homogenization buffer (1 mM dithiothreitol, 0.1 mM leupeptin, 5 mM benzamidine, 10 μ g/ml soybean trypsin inhibitor, 5 μ g/ml aprotinin, 2 μ M pepstatin A and 2 mM phenylmethylsulfonyl fluoride in 12.5 mM Tris, 1.25 mM EGTA, 1.25 mM EDTA and 0.25% Triton X 100, pH 7.6). Islets were then disrupted by sonic oscillation. Duplicate aliquots were analyzed for protein content with the Lowry assay (Lowry *et al.* 1951) using BSA to generate the standard curve and a rat liver preparation in homogenization buffer as an internal standard. Thirty micrograms of CD-1 islet protein and 20 μ g of control 3T3 cell lysate were separated by SDS-PAGE electrophoresis using a 4% stacking gel with a 7% separating gel at 12 mA and 16 mA respectively. Separated proteins were electro-transferred onto a polyvinylidene difluoride transfer membrane with 15 V for 20 h. Transfer was confirmed by staining with Ponceau S. Membranes were then briefly washed with distilled deionized water. PI3K, IRS-1 and IR β subunit membranes were blocked in Blotto A (Tris-buffered saline containing 5% Carnation non-fat dried milk and 0.05% Tween 20) for 1 h 50 min. IRS-2 membranes were blocked in PBS (pH 7.1 with 3% Carnation non-fat dried milk). For PI3K, membranes were incubated for 90 min with anti PI3K rabbit antisera (1:2000 dilution), washed, followed by incubation for 45 min with anti rabbit IgG-horseradish peroxidase (HRP) (0.5 μ g/ml) and then washed again. For IRS-1, membranes were incubated for 2 h with primary antibody, rabbit polyclonal IgG anti IRS-1 (1 μ g/ml), washed, followed by incubation for 90 min with anti rabbit IgG-HRP (0.5 μ g/ml) and then washed again. For IR β subunit, membranes were incubated for 2 h with anti IR β subunit rabbit IgG (1 μ g/ml), washed, followed by incubation for 90 min with anti rabbit IgG-HRP (0.5 μ g/ml) and then washed again. For IRS-2, membranes were incubated for 2 h with primary antibody, rabbit polyclonal IgG anti IRS-2 (1.0 μ g/ml), washed, followed by incubation for 90 min with anti rabbit IgG-HRP (0.5 μ g/ml) and then washed again. Antigen-antibody complexes were visualized using the NEN Western blot Chemiluminescence Reagent *Plus* system (Perkin Elmer, Boston, MA, USA). For PI3K and IR β subunit, membranes were exposed for 1 min while for IRS-1 and IRS-2 membranes

were exposed for 2 min on the Kodak Image Station 440cf. Images were captured and transferred to the Kodak 1D Image Analysis software for further analysis.

Reagents

Hank's solution was used for the islet isolation. The perfusion medium consisted of 115 mM NaCl, 5 mM KCl, 2.2 mM CaCl_2 , 1 mM MgCl_2 , 24 mM NaHCO_3 and 0.17 g/dl BSA. The ^{125}I -labeled insulin for the insulin assay was purchased from New England Nuclear. BSA (RIA grade), glucose, carbachol, α -KIC, wortmannin, LY294002 and the salts used to make the Hank's solution and perfusion medium were purchased from Sigma (St Louis, MO, USA). Forskolin was obtained from Calbiochem (La Jolla, CA, USA). Nitrendipine was the gift of A Scriabine of the Miles Institute for Preclinical Pharmacology (Elkhart, IN, USA). Rat insulin standard (lot no. 615-ZS-157) was the generous gift of Dr Gerald Gold, Eli Lilly Co. (Indianapolis, IN, USA). Collagenase (Type P) was obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN, USA). Anti rabbit IgG, anti β -subunit of the IR and anti IRS-1 were obtained from Santa Cruz Biotechnology, Inc, Santa Cruz, CA, USA. Anti IRS-2, anti PI3-K and the 3T3 cell lysates (positive controls for the IR, IRS-1, IRS-2 and PI3K) were obtained from Upstate Biotechnology, Lake Placid, NY, USA.

Statistics

Statistical significance was determined using the Student's *t*-test for unpaired data or analysis of variance in conjunction with the Newman-Keuls test for unpaired data. A *P* value ≤ 0.05 was taken as significant. Values presented in the figures and Results represent means \pm S.E. of at least three observations.

Results

Responses of rat or mouse islets to 10 mM glucose

As shown in Fig. 1, rat islets respond to sustained 10 mM glucose stimulation with a brisk biphasic insulin secretory response characterized by a large and rising second phase response. In the presence of 3 mM glucose, prestimulatory release rates of 50 ± 6 pg/islet per min were measured. In response to 10 mM glucose, peak first phase release rates averaged 93 ± 14 pg/islet per min. Secretion rates rose sharply as the perfusion progressed and 40, 50 or 60 min after the onset of stimulation averaged 603 ± 11 , 560 ± 34 or 554 ± 25 pg/islet per min respectively ($n=3$). If one takes into account the steady decline in release rates from rat islets maintained at 3 mM glucose alone for 90 min (29 ± 3 pg/islet per min ($n=3$), results not shown), an approximately 15- to 20-fold increase in second phase

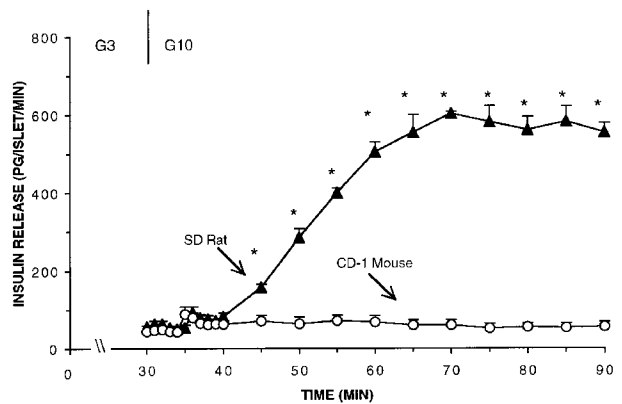


Figure 1 Glucose-induced release from perfused rat and mouse islets: studies with 10 mM glucose. Groups of 14–18 rat (\blacktriangle , $n=3$) or mouse (\circ , $n=7$) islets were isolated and perfused. For the initial 30 min the islets were maintained with 3 mM glucose (G3) to establish basal and stable insulin secretory rates. Islets were then perfused (indicated by the vertical line) for 60 min with 10 mM glucose (G10). Mean values \pm S.E. are given. The asterisks indicate a significant ($P < 0.05$) difference between release values at these time-points. This and subsequent perfusion figures have not been corrected for the dead space in the perfusion apparatus, 2.5 ml or 2.5 min with a flow rate of 1 ml/min.

response to 10 mM glucose stimulation was noted. This increment in glucose responsiveness compares favorably with that observed in studies using the perfused pancreas preparation (Gerich *et al.* 1974, Pagliara *et al.* 1974, Grill *et al.* 1978, O'Conner *et al.* 1980).

In response to 10 mM glucose stimulation, peak first phase release rates from mouse islets averaged 89 ± 18 pg/islet per min ($n=7$) (Fig. 1), a value similar to that observed from rat islets in response to the hexose. However, at a time when release rates from rat islets were increased about 15- to 20-fold, the response from mouse islets was minimal. For example 40, 50 or 60 min after the onset of 10 mM glucose stimulation release rates from mouse islets averaged 60 ± 13 , 56 ± 10 or 56 ± 13 pg/islet per min respectively. Compared with release rates of 27 ± 4 pg/islet per min ($n=3$, results not shown) from mouse islets maintained for 90 min at 3 mM glucose, an approximately twofold increase in release rates was observed. This lack of response cannot be ascribed to insulin deficiency since exposure of these same islets to 20 mM glucose plus 10 μM carbachol was accompanied by a dramatic sustained increase in insulin release. For example, 15–20 min after the onset of 20 mM glucose plus 10 μM carbachol stimulation release rates increased to 671 ± 85 pg/islet per min (Fig. 2).

Mouse islet responses to wortmannin

Mouse islet responses to the PI3K inhibitor wortmannin were investigated next. The concentration (50 nM) of wortmannin employed in these studies has been shown

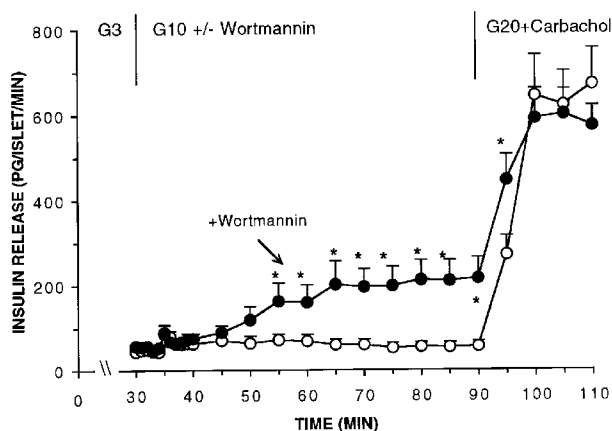


Figure 2 Effect of wortmannin on 10 mM glucose-induced insulin release from mouse islets. Groups of mouse islets were perfused for 30 min with 3 mM glucose and for an additional 60 min with 10 mM glucose alone (\circ , $n=7$, these are the same mouse islet data as presented in Fig. 1) or in the additional presence of 50 nM wortmannin (\bullet , $n=6$). For the final 20 min of the perfusion both groups of islets were stimulated with 20 mM glucose plus 10 μ M carbachol. Mean values \pm s.e. are given. The asterisks indicate significance ($P<0.05$) between release rates measured in the presence or absence of wortmannin at these time-points.

previously by us to stimulate glucose-induced secretion about threefold (Zawalich & Zawalich 2000). Studies by several groups using rat islets, β TC3 or MIN6 cells have demonstrated that this level of wortmannin significantly inhibits PI3K activity between 60 and 90% (Hagiwara *et al.* 1995, Gao *et al.* 1996). The results are presented in Fig. 2. The addition of the fungal metabolite markedly enhanced the responses of CD-1 mouse islets to 10 mM glucose, particularly as the perfusion progressed. For example, release rates from islets stimulated with 10 mM glucose alone averaged 60 ± 13 , 56 ± 10 or 56 ± 13 pg/islet per min 40, 50 or 60 min after the onset of 10 mM glucose stimulation respectively. The responses in the presence of 50 nM wortmannin averaged 195 ± 43 , 211 ± 47 or 215 ± 49 pg/islet per min ($n=6$) respectively, with all values being significantly ($P<0.05$) greater than control responses.

In response to 20 mM glucose stimulation alone (Fig. 3), peak first phase release rates averaged 121 ± 15 pg/islet per min ($n=12$). Sixty minutes after the onset of stimulation release rates averaged 70 ± 12 pg/islet per min. The inclusion of 50 nM wortmannin had no significant effect on peak first phase release rates (150 ± 11 pg/islet per min, $n=6$). However, second phase secretory responses were significantly increased (Fig. 3). Stimulating both groups of islets with 20 mM glucose plus 10 μ M carbachol for the final 20 min of the perfusion resulted in comparable rates of secretion.

Additional studies were conducted with mouse islets pretreated with 50 nM wortmannin during the 30-min perfusion with 3 mM glucose. In this case, the potentiat-

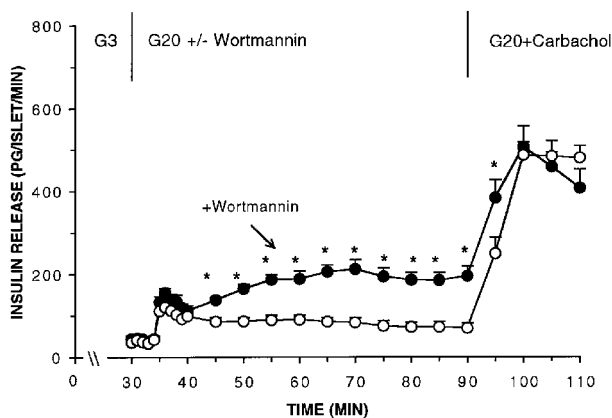


Figure 3 Effect of wortmannin on 20 mM glucose-induced insulin release. Groups of mouse islets were perfused for 30 min with 3 mM glucose and for an additional 60 min with 20 mM glucose alone (\circ , $n=12$) or in the additional presence of 50 nM wortmannin (\bullet , $n=6$). For the final 20 min of the perfusion both groups of islets were stimulated with 20 mM glucose plus 10 μ M carbachol. Mean values \pm s.e. are given. The asterisks indicate significance ($P<0.05$) between release rates measured in the presence or absence of wortmannin. At least four experiments were conducted under each condition.

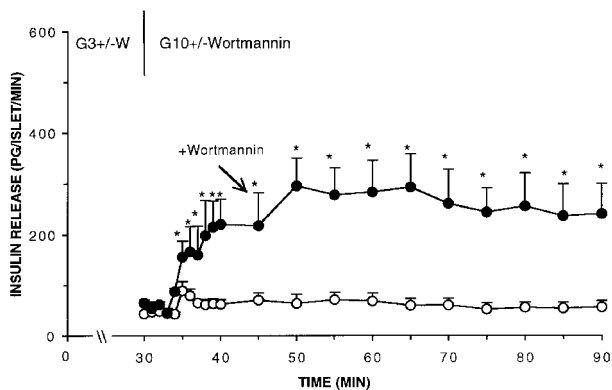


Figure 4 Pretreating CD-1 mouse islets with wortmannin accelerates potentiated responses to 10 mM glucose. One group of mouse islets was perfused for 30 min with 3 mM glucose and for an additional 60 min with 10 mM glucose alone (\circ , $n=7$, these are the same mouse islet data as presented in Fig. 1). The second group (\bullet , $n=4$) was similarly treated except that 50 nM wortmannin (W) was included during the entire perfusion. Mean values \pm s.e. are given. The asterisks indicate significance ($P<0.05$) between release rates measured in the presence or absence of wortmannin at these time-points.

ing effects of the fungal metabolite on secretion in response to 10 mM glucose stimulation were evident at earlier times (Fig. 4).

Effects of nitrendipine on wortmannin-potentiated secretion

Blocking calcium influx into the β -cell abolishes glucose-induced insulin secretion (Henquin *et al.* 1982). In the next series of experiments, islets were perfused and

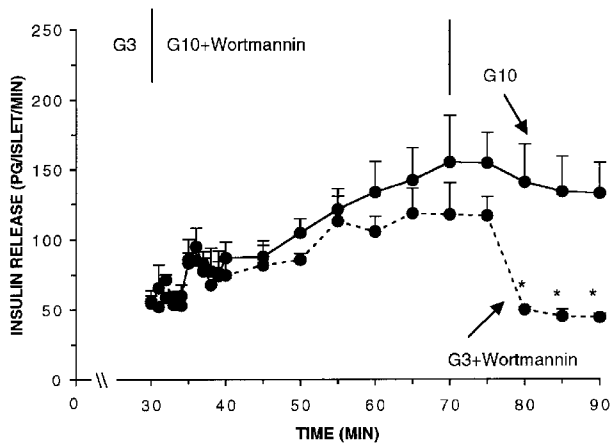


Figure 5 Examining the reversibility of wortmannin's effect on glucose-induced release. Two groups of mouse islets were studied. They were both perfused for 30 min with 3 mM glucose and for an additional 40 min with 10 mM glucose plus 50 nM wortmannin (indicated by the vertical line). For the final 20 min, one group was perfused with 10 mM glucose alone (solid line, $n=4$) while the second group was perfused with 3 mM glucose plus 50 nM wortmannin (broken line, $n=4$). Note the rapid decline in secretion when the glucose level was lowered to 3 mM but not when wortmannin was omitted from the medium in the continued presence of 10 mM glucose. Mean values \pm s.e. are given. The asterisks indicate a significant ($P<0.05$) difference between release values at this time.

stimulated with the combination of 10 mM glucose, 50 nM wortmannin plus 500 nM of the calcium channel influx inhibitor nitrendipine. The inclusion of the calcium channel antagonist blocked insulin secretion to the combination of 10 mM glucose plus wortmannin. For example, in the absence of nitrendipine, release rates in response to glucose plus wortmannin averaged 195 ± 43 , 211 ± 47 or 215 ± 49 pg/islet per min ($n=6$) 40, 50 or 60 min after the onset of stimulation respectively (Fig. 2). In the additional presence of nitrendipine, release rates at these times averaged 33 ± 5 , 41 ± 7 or 35 ± 2 pg/islet per min ($n=3$) respectively (results not shown).

Reversibility of wortmannin

Mouse islets were stimulated for 40 min with 10 mM glucose plus 50 nM wortmannin (Fig. 5). Consistent with previous findings, release rates increased. Forty minutes after the onset of stimulation, wortmannin was omitted from the perfusion medium (the glucose concentration was clamped at 10 mM) or the glucose level was lowered to 3 mM (the wortmannin concentration was clamped at 50 nM). Increased rates of insulin release persisted in spite of the removal of the fungal metabolite from 10 mM glucose-containing perfusion medium. However, in spite of the continued presence of 50 nM wortmannin, release rates abruptly subsided to prestimulatory values when the glucose level was lowered to 3 mM (Fig. 5).

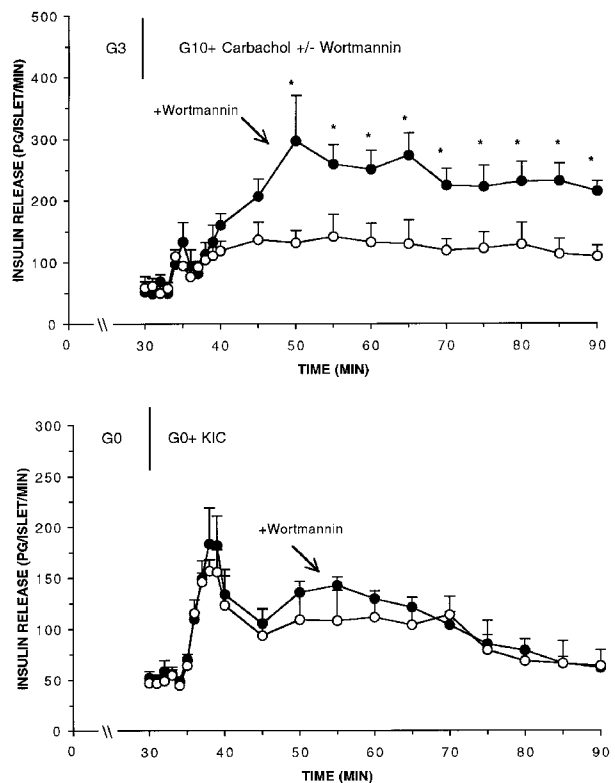


Figure 6 Wortmannin amplifies release in response to glucose plus carbachol but not to α -ketoisocaproate (KIC). Groups of mouse islets were perfused for 30 min with 3 mM glucose. (Top) For the next 60 min (onset indicated by vertical line) islets were stimulated with 10 mM glucose plus 1 μ M carbachol (\circ , $n=4$) or 10 mM glucose plus 1 μ M carbachol plus 50 nM wortmannin (\bullet , $n=4$). (Bottom) For the next 60 min islets were stimulated (indicated by vertical line) with 10 mM KIC alone (\circ , $n=5$) or 10 mM KIC plus 50 nM wortmannin (\bullet , $n=5$). Mean values \pm s.e. are given. The asterisks indicate a significant ($P<0.05$) difference between release values at these time-points.

Specificity of wortmannin

Wortmannin amplified insulin release from mouse islets stimulated with 10 mM glucose plus 1 μ M carbachol (Fig. 6, top). However, when used at a stimulatory concentration approximately equipotent to 10 mM glucose, 50 nM wortmannin had no effect on release stimulated by 10 mM α -KIC (Fig. 6, bottom).

Effects of LY294002 on 10 mM glucose-induced release from mouse islets

The inhibitory effects of wortmannin on PI3K activity can be duplicated by a structurally distinct compound, LY294002 (Vlahos *et al.* 1994). If the amplifying effects of wortmannin on 10 mM glucose-induced release from mouse islets is due to PI3K inhibition, then it might be predicted that LY294002 should have a similar

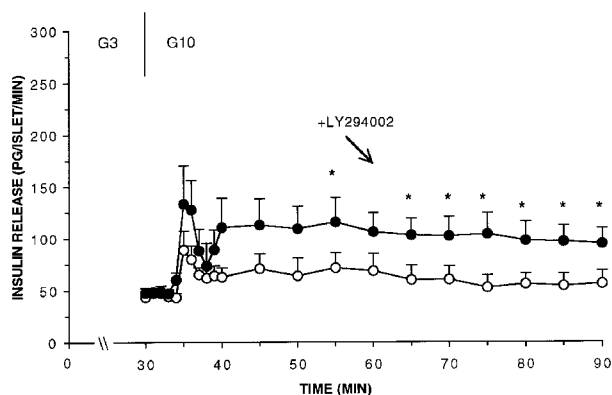


Figure 7 LY294002 potentiates 10 mM glucose-induced insulin secretion. Groups of 14–18 mouse islets were isolated and perfused. For the initial 30 min all islets were maintained with 3 mM glucose to establish basal and stable insulin secretory rates. Islets were then perfused (indicated by the vertical line) for 60 min with 10 mM glucose alone (\circ , $n=7$, these are the same data as in Fig. 2) or 10 mM glucose plus 10 μ M LY294002 (\bullet , $n=5$). Mean values \pm S.E. are given. The asterisks indicate a significant ($P<0.05$) difference between release rates at this time.

potentiating effect. This was investigated next. LY294002 (10 μ M) potentiated 10 mM glucose-induced release (Fig. 7). The stimulatory effect of LY294002 was smaller than that observed with 50 nM wortmannin (compare Figs 2 and 7).

Effects of wortmannin on release from ob/ob mouse islets

A variety of mouse models have been employed to probe the nature of the insulin signaling systems that regulate glucose homeostasis. Prominent among these is the ob/ob mouse model of hyperinsulinemia, insulin resistance and obesity. It has been reported that signaling via the PI3K pathway is markedly impaired *in vivo* in these animals, a biochemical lesion thought to play an important role in their insulin resistance (Folli *et al.* 1993, Kerouz *et al.* 1997). The impact of wortmannin on release from ob/ob mouse islets or their lean littermates was determined. Like islets isolated from CD-1 mice, islets isolated from lean C57BL donors were sensitive to the augmenting effect of 50 nM wortmannin (Fig. 8, top). For example, while insulin release rates from control islets stimulated with 10 mM glucose alone averaged 44 ± 5 , 36 ± 3 or 27 ± 4 pg/islet per min ($n=3$) 40, 50 or 60 min after the onset of stimulation, the inclusion of 50 nM wortmannin significantly ($P<0.05$) increased the responses at these times to 85 ± 10 , 94 ± 12 or 99 ± 10 pg/islet per min ($n=4$) respectively.

We next examined the actions of wortmannin on 10 mM glucose-induced insulin release rates from ob/ob mouse islets. As previously reported (Chen *et al.* 1993, Zawalich & Zawalich 1996b, Chen & Romsos 1997), islets isolated from these animals were significantly more

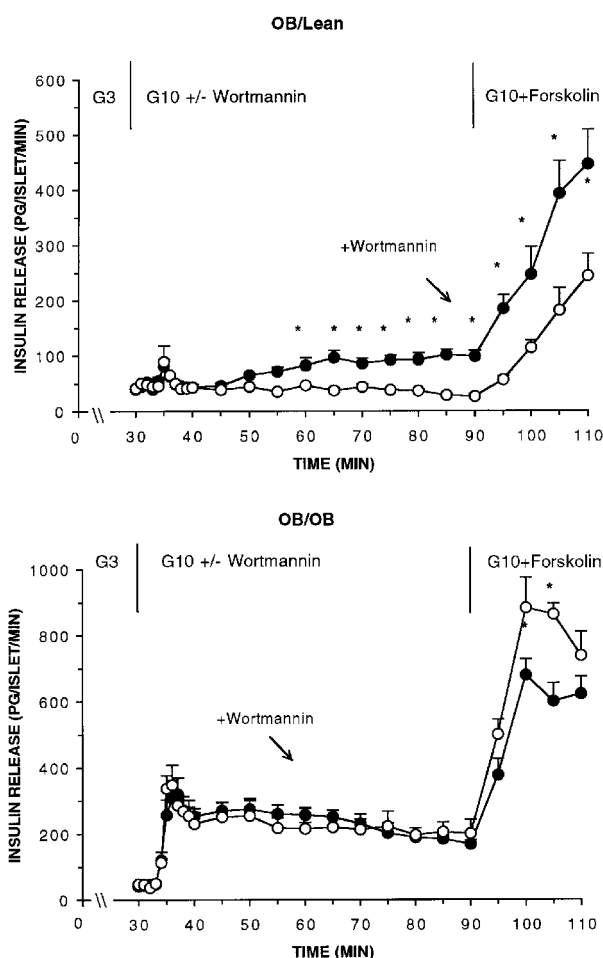


Figure 8 Effect of wortmannin on 10 mM glucose-induced insulin release from islets of lean or ob/ob mice. (Top) Two groups of 14–18 islets were isolated from lean littermates of ob/ob mice and perfused for 30 min with 3 mM glucose. One group (\circ , $n=4$) was then stimulated with 10 mM glucose alone for 60 min. The second group (\bullet , $n=4$) was stimulated with 10 mM glucose plus 50 nM wortmannin for 60 min. For the final 20 min both groups were perfused with 10 mM glucose plus 5 μ M forskolin. Mean values \pm S.E. are given. The asterisks indicate a significant ($P<0.05$) difference between release values at this time. (Bottom) Two groups of 14–18 islets were isolated from ob/ob mice and perfused for 30 min with 3 mM glucose. One group (\circ , $n=5$) was then stimulated with 10 mM glucose alone for 60 min. The second group (\bullet , $n=5$) was stimulated with 10 mM glucose plus 50 nM wortmannin. For the final 20 min both groups were perfused with 10 mM glucose plus 5 μ M forskolin. Mean values \pm S.E. are given. The asterisks indicate a significant ($P<0.05$) difference between release values at this time. Note the change in scale between top and bottom panels.

responsive to 10 mM glucose than islets from their lean counterparts (compare open circles in Fig. 8 (top) with open circles in Fig. 8 (bottom)). Both phases of release were amplified from ob/ob mouse islets although the kinetics of the response, a spike of first phase insulin

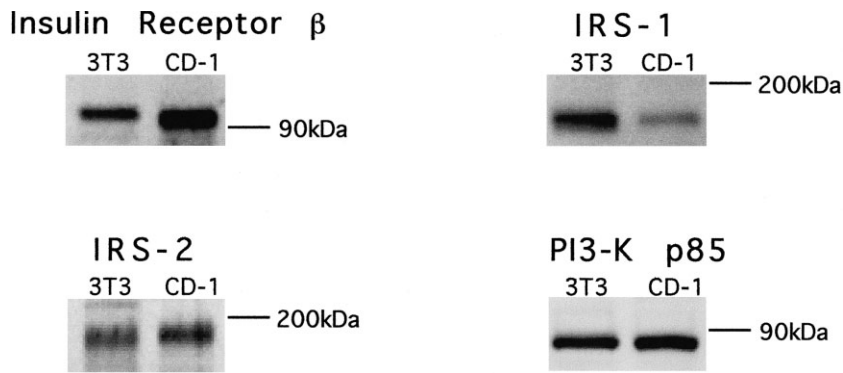


Figure 9 CD-1 mouse islets express insulin receptor (IR), IR substrate (IRS)-1, IRS-2 and phosphatidylinositol 3-kinase (PI3K). Groups of CD-1 mouse islets were sonicated, Western blotted (30 μ g protein/lane) and probed for the presence of insulin signaling compounds. The IR β subunit was detected at 93 kDa, IRS-1 at 175 kDa, IRS-2 at 185 kDa and the PI3K p85 subunit at 85 kDa. Control cell lysates from 3T3 cells (20 μ g/lane, left lane in all blots) were similarly treated. Two to three separate experiments were performed under each condition and a representative blot is shown.

secretion followed by a sustained but flat second phase response, were similar to those of lean islets. When stimulated with 10 mM glucose in the presence of 50 nM wortmannin, islets from ob/ob mice, in contrast to lean CD-1 mouse islets or lean C57BL mouse islets, were immune to the potentiating effect of the fungal metabolite. At no time-point during the perfusion was any amplifying effect observed.

Western blot analyses

The concept that wortmannin exerts its effects on β -cell responses via inhibition of PI3K signaling would be supported by the demonstration that insulin signaling components are present in our islet preparations. To this end, CD-1 mouse islet homogenates were Western blotted and probed for the IR, IRS-1, IRS-2 and PI3K. As shown in Fig. 9, when compared with control cell lysates, strong bands corresponding to the IR, IRS-2 and PI3K were detected in CD-1 mouse islets while a considerably lesser signal was detected for IRS-1.

Discussion

We reported previously that insulin secretion from rat islets in response to 8 mM glucose stimulation was dramatically potentiated by 50 nM wortmannin, a fungal metabolite that potently inhibits PI3K at the level employed in these studies (Okada *et al.* 1994, Hagiwara *et al.* 1995, Gao *et al.* 1996). This effect on insulin release led us to suggest that PI3K activity in β -cells may provide a communications link between islets and peripheral tissues. In this context we speculated that, as peripheral tissues become resistant to circulating insulin as a consequence of

reduced signaling via the PI3K pathway (Kim *et al.* 1999, Cusi *et al.* 2000), the same biochemical perturbation at the level of the β -cell evokes a linked and compensatory insulin secretory response from these cells. We also predicted that wortmannin should be less effective in stimulating release from islets of Zucker fatty rats, a model of hyperglycemia, hyperinsulinemia and insulin resistance in which *in vivo* reductions in PI3K activity have been demonstrated (Anai *et al.* 1998). This prediction was confirmed (Zawalich & Zawalich 2000).

These initial studies using rat islets to study the inter-relationships between PI3K inhibition by wortmannin and the secretion of insulin from β -cells prompted us to consider similar experiments with freshly isolated and perfused mouse islets. This was done for several reasons. First, since mouse islet responses to glucose stimulation deviate from rat islets in several major ways (Berglund 1987, Zawalich *et al.* 1995b), we considered it prudent to establish whether this anomalous behavior to glucose might be extended to wortmannin as well. Secondly, studies using HIT cells (Straub & Sharp 1996) treated with wortmannin have suggested that PI3K activation may play a stimulatory role in the insulin secretory process, while studies with cultured neonatal rat monolayer pancreatic islets (Zhao *et al.* 1998) suggest that PI3K activation mediates leptin-induced inhibition of secretion. However, in terms of their sensitivity to glucose stimulation, the preparations employed in these previous studies deviate from normal islets. Thirdly, transgenic and knockout mice are being increasingly employed to probe the contribution of insulin signaling pathway lesions to the regulation of glucose homeostasis (Araki *et al.* 1994, Brüning *et al.* 1997, Kulkarni *et al.* 1999a). Fourthly, disruption of insulin signaling often culminates in hyperinsulinemia, a result that might be predicted if insulin functions normally to

restrain insulin output from them (Araki *et al.* 1994, Tamemoto *et al.* 1994, Brüning *et al.* 1997).

Due in part to the small amounts of islet tissue available for investigation and the large numbers required to accurately measure PI3K activity (Gao *et al.* 1996), we did not directly measure the inhibition of PI3K by wortmannin. However, the wortmannin levels employed in these experiments are exactly the same as those used to inhibit the enzyme in a large number of preparations including β -cells (Acaro & Wymann 1993, Gao *et al.* 1994, Okada *et al.* 1994, Hagiwara *et al.* 1995, Hausdorff *et al.* 1999). Islets were collagenase isolated and immediately perfused to assess the kinetics and amplitude of their insulin secretory responses to glucose. Islets were not cultured prior to study. This approach was employed for several reasons. First, statically incubated islets may not be optimal for this type of analysis. This preparation yields no information on the dynamics of release. Secondly, statically incubated islets are vulnerable to the effects of products that accumulate in the medium and that appear to negatively influence insulin release from them (Loreti *et al.* 1974, Ammon & Verspohl 1976, Easom *et al.* 1997). Thirdly, short-term culture, of rat islets at least, diminishes their secretory responses to glucose stimulation (Malaisse-Lagae *et al.* 1987, Metz 1988, Zawalich *et al.* 1995a) and by using freshly studied islets we avoided the potential introduction of any artifacts induced by culturing.

These studies with mouse islets parallel those we previously reported using rat islets (Zawalich & Zawalich 2000) and their conduct was motivated by previous reports demonstrating differences to glucose stimulation between these two species (Berglund 1980, Ma *et al.* 1995, Zawalich *et al.* 1995b). We first confirmed that these differences to glucose stimulation exist under the conditions employed in these experiments.

The next set of experiments was conducted with wortmannin. A concentration of 50 nM was employed for these studies since, at this level, maximal effects on glucose-induced release from rat islets were noted previously (Zawalich & Zawalich 2000). While profound differences in their sensitivity to glucose stimulation exist, mouse islets like rat islets were sensitive to the potentiating effects of wortmannin on glucose-induced release. In response to 10 mM glucose plus wortmannin, secretion was maximally increased by three- to four-fold above release rates observed in the presence of 10 mM glucose alone. No effect of wortmannin on release was observed if the glucose level was maintained at 3 mM. In addition, while the augmenting effect of wortmannin on 10 mM glucose persisted even after its removal from the medium, lowering the glucose level to 3 mM resulted in a rapid abatement of release. If wortmannin specifically inhibits PI3K at the level employed, these findings suggest that PI3K activation restrains the impact of a glucose-derived metabolic signal on the secretory process.

Several other aspects of wortmannin's effects on islet responses were investigated. First, the effects of the fungal metabolite are dependent on calcium influx, since the calcium channel antagonist nitrendipine abolished release to the hexose plus wortmannin. This, and its glucose dependency, would seem to preclude non-specific β -cell damage and the subsequent leakage of insulin into the perfusion medium as the cause of wortmannin's effects. Secondly, wortmannin further potentiated the secretory responses of mouse islets to stimulation by the combination of glucose plus carbachol. This combination of agonists was selected since meal-induced insulin release appears to be regulated, at least in part, by both glucose and vagally derived cholinergic signals (Loubatieres-Mariani *et al.* 1973, Louis-Sylvestre 1976, 1978, Zawalich *et al.* 1989a). Finally, and potentially important with regards to its potential mechanism of action, is the finding that wortmannin had no effect on α -KIC-induced secretion. This deamination product of leucine metabolism is exclusively metabolized within the mitochondria (Panten *et al.* 1974, Panten 1975).

We duplicated the potentiating effects of PI3K inhibition on β -cell responses to glucose by using a structurally distinct inhibitor of the enzyme, LY294002 (Vlahos *et al.* 1994). This finding lends further credence to the concept that inhibition of PI3K plays a role, at least in part, in the amplified secretory responses observed from mouse islets to 10 mM glucose stimulation.

We next attempted to establish the potential *in vivo* physiologic and pathophysiologic relevance of these *in vitro* observations. For this we employed islets isolated from ob/ob mice. These animals are characterized by obesity, hyperglycemia, insulin resistance and hyperinsulinemia (Kahn 1980). Pertinent to the next series of experiments using islets isolated from them is the observation that *in vivo* signaling via PI3K is also impaired in liver and muscle from these animals (Kerouz *et al.* 1997) and that islets isolated from ob/ob mice are resistant to the negative feedback effect that insulin exerts on its own secretion (Loreti *et al.* 1974). Based on previous studies (Loreti *et al.* 1974, Soll *et al.* 1974, Kerouz *et al.* 1997), we theorized that the reduction in PI3K activity demonstrated in liver and muscle might not be uniquely confined to these tissues but might also be present in ob/ob islets. If this is an accurate portrayal of *in vivo* biochemical events, then it might be predicted that islets isolated from ob/ob mice, in which PI3K activity may be already reduced, would be less sensitive to the potentiating effect of PI3K inhibition by wortmannin. The next experiments focused on this issue.

As a precondition to the conduct of these studies, it was imperative initially to address three important issues. First, we had to establish that islets from lean littermates of ob/ob mice, like CD-1 mice, are relatively insensitive to 10 mM glucose stimulation alone. Secondly, we had to establish that islets from lean C57BL mice are sensitive

to the potentiating effects of wortmannin. Thirdly, we had to confirm that islets from ob/ob mice are hyper-responsive to glucose stimulation *in vitro* as they are *in vivo*. The results of these experiments are as follows: (1) when stimulated with 10 mM glucose alone, and in contrast to rat islet responses but similar to that observed with islets isolated from CD-1 mice, lean C57BL mice are minimally responsive to 10 mM glucose stimulation; (2) however, wortmannin potentiated 10 mM glucose-induced secretion from this strain of mice as it does in CD-1 mice and rats; (3) confirming previous studies (Chen *et al.* 1993, Zawalich & Zawalich 1996b, Chen & Romsos 1997), islets from ob/ob mice are hyper-responsive to glucose stimulation during a dynamic perfusion *in vitro*. The fulfillment of these three conditions led us to conduct studies using ob/ob islets stimulated with glucose plus wortmannin.

When stimulated with 10 mM glucose plus 50 nM wortmannin, a combination that results in a threefold potentiation of secretion from control CD-1 or C57BL mice, no amplifying effect on 10 mM glucose-induced secretion was observed from ob/ob mouse islets. It thus appears that the reduced activity of PI3K documented to occur *in vivo* in several insulin-dependent tissues (Folli *et al.* 1993, Kerouz *et al.* 1997) may occur in their β -cells as well, is maintained during the islet isolation procedure, and is responsible, at least in part, for the augmented *in vivo* and *in vitro* secretory responses of ob/ob mouse islets to glucose stimulation and also for ob/ob islet insensitivity to wortmannin. The findings presented here with wortmannin and a previous report demonstrating that islets from ob/ob mice are resistant to the negative feedback effect that insulin exerts on its own secretion (Loreti *et al.* 1974) also suggest a common biochemical explanation: reduced β -cell PI3K activity. While other factors, including islet hypertrophy, have to be considered as contributing to this amplified secretory response to glucose, the fact that wortmannin sensitivity is abolished in these animals points to a potentially important role for disrupted PI3K activation.

Disruption of insulin signaling pathways has been reported in knockout mice with consistent effects on the β -cell. For example, Tamemoto *et al.* (1994) found significantly higher plasma insulin levels in mice with targeted disruption of IRS-1 gene when compared with control animals. Plasma glucose levels were comparable and thus could not account for the hyperinsulinemia. Similar findings were reported by Araki *et al.* (1994). Mice double heterozygous for null alleles in the IR and IRS-1 genes develop marked hyperinsulinemia despite no significant changes in plasma glucose concentrations from fed animals (Brüning *et al.* 1997). Mice with combined heterozygous null mutations in the IR, IRS-1 and/or IRS-2 are characterized by both fed and fasting hyperinsulinemia with little if any alteration in plasma glucose levels (Kido *et al.* 2000). While β -cell hyperplasia un-

doubtedly contributes to hyperinsulinemia in these genetically altered mice, this effect is variable and does not always correlate with the degree of hyperinsulinemia (Brüning *et al.* 1997, Kido *et al.* 2000). Most recently, Eto *et al.* (2002) reported that islets isolated from mice deficient in the p85 regulatory subunit of PI3K exhibit augmented insulin secretory responses to glucose stimulation. These findings and the present studies suggest that a reduction in the negative feedback effect that insulin exerts upon its own secretion from the β -cell (Iversen & Miles 1971, Ammon & Verspohl 1976, Liljenquist *et al.* 1978, Ammon *et al.* 1991) may play a role in their *in vitro* hyper-sensitivity to glucose and their *in vivo* development of hyperinsulinemia.

Several studies have examined the effects of β -cell specific alterations in the IR and IRS-1 (Kulkarni *et al.* 1999a,b) on β -cell function. Tissue-specific knockout of the IR results in a reduction in glucose-stimulated insulin secretion *in vivo*. Arginine-induced release was unaffected (Kulkarni *et al.* 1999a). In contrast, both glucose- and arginine-induced release *in vivo* is impaired in IRS-1-deficient mouse islets (Kulkarni *et al.* 1999b). After a 48-h culture period in Dulbecco's minimum essential medium (DMEM), impaired responses to glucose stimulation in batch-incubated islets isolated from IRS-1-deficient animals were also noted. For at least several reasons it is difficult to compare these findings with ours. First, our studies were acute in nature. Long-term reductions in insulin signaling might induce unique β -cell alterations not evident in short-term perfusion studies. Secondly, our islets were studied immediately after isolation. In rat islets, at least, short-term culturing results in profound reductions in β -cell function (Malaisse-Lagae *et al.* 1987, Metz 1988, Zawalich *et al.* 1995a). Whether similar changes occur in mouse islets cannot be excluded. Thirdly, our islets were studied during a dynamic perfusion. The excessive accumulation of potentially inhibitory factors is thus mitigated. Finally, our islets were stimulated in a KRB medium supplemented with one or at most two agonists for release. DMEM contains multiple amino acids with established cumulative effects on release that may influence the secretory responses to glucose. Their possible contribution to the secretory findings made has to be considered.

Based on islet volume and insulin secretory rates, it has been estimated that during maximal glucose stimulation insulin bathing the β -cell approaches 100–200 nM (Zawalich *et al.* 1975), a level at least 1000-fold greater than systemic concentrations of the hormone (Del Prato *et al.* 1994, Robinson *et al.* 1998). Even under non-stimulatory conditions, the level of insulin bathing the β -cell must be far in excess of that bathing other insulin-sensitive tissues such as liver, fat and muscle. For heuristic purposes at least, it might be proposed that these high levels of insulin exert a tonic negative feedback effect on insulin secretion via PI3K activation. This schema would explain why disruption of PI3K signaling by wortmannin

amplifies glucose-induced insulin secretion. Failure of this normal negative feedback arrangement would be predicted to result in amplified secretory responses to glucose and may explain not only the hyperinsulinemia in obese animals such as the Zucker fatty rat and the ob/ob mouse but also the failure of wortmannin to further amplify secretion from islets of these animals (Zawalich & Zawalich 2000). Yet to be established, however, is whether the disruption of PI3K signaling in β -cells of obese rats and mice is caused by hyperinsulinemia and/or hyperglycemia (Pillay *et al.* 1996).

To summarize, we have confirmed that CD-1 mouse islets contain the requisite insulin signaling proteins, IR, IRS-1, IRS-2 and PI3K, characterized in other insulin-dependent tissues. Secondly, islets isolated from several strains of control mice exhibit a profoundly reduced insulin secretory response to 10 mM glucose stimulation when compared with rat islets studied under identical conditions. We have demonstrated that islets isolated from several strains of lean control mice, like islets isolated from lean rats, are sensitive to the potentiating effect of wortmannin. We have shown that islets isolated from ob/ob mice, an animal model characterized by insulin resistance, hyperinsulinemia, hyperglycemia and impaired PI3K activity *in vivo*, are insensitive to the amplifying effects of wortmannin *in vitro*. These mice are also less sensitive to the negative feedback effect that insulin exerts on its own secretion (Loreti *et al.* 1974). In this regard, the findings made with ob/ob mice are similar to our previous results obtained using perfused islets isolated from insulin-resistant, hyperinsulinemic Zucker fatty rats (Zawalich & Zawalich 2000). Thus, the *in vivo* status of the donor animals' insulin signaling pathways appears critical in determining *in vitro* islet sensitivity to wortmannin irrelevant of the animal model employed. Finally, these results suggest that the hyperinsulinemia resulting from insulin resistance may be a consequence of a similar biochemical lesion in liver, muscle, adipose and β -cells – a reduction in signaling via PI3K. This elegant control mechanism thus ensures that the release of insulin from the β -cell will be titrated to the degree of insulin resistance.

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