Inhibition of Lipase Activity and Lipolysis in Rat Islets Reduces Insulin Secretion

Hindrik Mulder,1 Shumin Yang,1 Maria Sörhede Winzell,1 Cecilia Holm,1 and Bo Ahlén2

Lipids may serve as coupling factors in KATP-independent glucose sensing in β-cells. We have previously demonstrated that β-cells harbor lipase activities, one of which is the hormone-sensitive lipase. Whether β-cell lipases are critical for glucose-stimulated insulin secretion (GSIS) by providing lipid-derived signals from endogenous lipids is unknown. Therefore, using a lipase inhibitor (orlistat), we examined whether lipase inhibition reduces insulin secretion. Islet lipolysis stimulated by glucose and diglyceride lipase activity was abolished by orlistat. Incubation of rat islets with orlistat dose dependently inhibited GSIS; this inhibition was reversed by 1 mmol/l palmitate, suggesting that orlistat acts via impaired formation of an acylglyceride-derived coupling signal. Orlistat inhibited the potentiating effect of forskolin on GSIS, an effect proposed to be due to activation of a lipase. In perfused islets, orlistat attenuated mainly the second phase of insulin secretion. Because the rise in islet ATP/ADP levels in response to glucose and oxidation of the sugar were unaffected by orlistat whereas the second phase of insulin secretion was reduced, it seems likely that a lipid coupling factor involved in KATP-independent glucose sensing has been perturbed. Thus, β-cell lipase activity is involved in GSIS, emphasizing the important role of β-cell lipid metabolism for insulin secretion.

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A large body of evidence suggests that lipids are required for appropriate glucose sensing in pancreatic β-cells. For instance, when triglycerides in pancreatic islets are depleted by hyperleptinemia, pancreatic β-cells fail to release insulin (1). Along similar lines, perfused pancreas from fasted rats is unresponsive to a rise in glucose (2). Importantly, in both cases, glucose responsiveness is reinstated by the addition of exogenous fatty acids.

The current view holds that lipids serve as coupling factors in glucose-stimulated insulin secretion (GSIS) that does not rely on closure of the ATP-sensitive K+ channel (KATP independent) (3). These putative mechanisms presumably drive the second phase of GSIS and accordingly have been termed as amplifying (4), and the coupling factors have been proposed to emanate from intermediary metabolism in the β-cell (5). Against this background, lipids have become attractive candidates for coupling factors in KATP-independent glucose sensing. In fact, a model has been proposed to delineate the combined events in metabolism of glucose and lipids that result in a dose-dependent regulation of insulin secretion, the so-called long-chain acyl-CoA hypothesis (3). This model is based on the observations that an increase in glucose metabolism in β-cells results in decreased oxidation of fats while esterification of lipids increases. As a consequence, the levels of complex lipids rise, perhaps acyl-CoA moieties, which then act as a signaling molecules coupling stimulus to secretion in the β-cell.

We have previously demonstrated that different preparations of β-cells exhibit lipase activities (6). Among these is the hormone-sensitive lipase (HSL), which, in white adipocytes, is the critical enzyme that hydrolyzes triglycerides to fatty acids (7). HSL is unique among lipases in that it is controlled by circulating hormones. Thus, hormones, such as glucagon, which is secreted in the fasted state, and adrenaline stimulate formation of cAMP in adipocytes. Consequently, protein kinase A (PKA) is activated and phosphorylates HSL on three serine residues (8), thereby activating the enzyme and controlling the rate of lipolysis. Insulin, however, is a potent inhibitor of lipolysis. This is achieved by activation of phosphodiesterase 3 B (9), which hydrolyzes cAMP, thereby inactivating PKA. In β-cells, however, the situation seems to be more complex. Although expression of HSL was confirmed using several techniques, activity assays indicated that lipases other than HSL may be in operation in β-cells (6). With regard to insulin secretion, it has been reported that the antilipolytic agent 3,5-dimethylpyrazole inhibits insulin secretion from isolated islets in response to glucose, cAMP-raising agents, and the mitochondrial fuel α-ketoisocaproic acid (10), suggesting a critical role for a lipase in β-cell stimulus-secretion coupling. However, observations from genetic mouse models, in which HSL has been inactivated, are in both support (11) and opposition (12) of a crucial role for HSL in control of insulin secretion. In sum, the studies available thus far suggest that lipases, other than HSL and that which remain to be identified, account for some lipase activity in islets and may play a role in β-cell stimulus-secretion coupling, whereas the role of HSL in these processes requires further clarification.

This study further explored whether β-cell lipase activity is required for GSIS. To circumvent the issue of which

From the 1Department of Cell and Molecular Biology, Lund University, Lund, Sweden; and the 2Department of Medicine, Lund University, Lund, Sweden. Address correspondence and reprint requests to Hindrik Mulder, MD, PhD, Section for Molecular Signaling, Department of Cell and Molecular Biology, Lund University, BMC C11 SE-221 84 Lund, Sweden. E-mail: hindrik.mulder@medkem.lu.se.

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8-Br-cAMP, 8-bromoadenosine-3',5'-cyclic monophosphate; AUC, area under the curve; GLP-1, glucagon-like peptide 1; GSIS, glucose-stimulated insulin secretion; HBSS, HEPES balanced salt solution; HSL, hormone-sensitive lipase; MOME, mono-oleoyl-2-O-mono-oleylglycerol; PKA, protein kinase A.

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particular lipase accounts for hydrolysis of acylglycerides in pancreatic β-cells, we used an inhibitor of a wide spectrum of lipases. Our choice was tetrahydrocolipstatin, orlistat, which is a lipophilic molecule that irreversibly binds to the catalytic site of a great number of lipases (13) and which has previously been shown to inhibit cAMP-induced insulin secretion in the clonal β-cell line HIT-T15 (14) and glucagon-like peptide 1 (GLP-1)-stimulated secretion in rat islets (15). The drug is widely used for treatment of obesity (16); it inhibits intestinal lipases, preventing uptake of lipids and thereby restricting caloric input. Orlistat, at therapeutic doses, is not taken up into the circulation, thereby precluding undesired systemic effects. By use of this tool in vitro, we were able to inhibit lipase activity in primary β-cells, thereby reducing lipolysis. These maneuvers resulted in an inhibition of insulin secretion, suggesting that intracellular mobilization of lipids via activation of lipases in β-cells is required for GSIS.

RESEARCH DESIGN AND METHODS

Reagents and animals. All reagents were from Sigma (St. Louis, MO) unless otherwise stated. Orlistat (tetrahydrocolipstatin) was supplied by Dr. Marcel K. Meier (Roche Pharmaceuticals, Basel, Switzerland). The drug was dissolved in DMSO/ethanol (60/40%) as a stock solution of 10 mmol/l. In all experiments, an identical concentration of the vehicle was added. Female Sprague-Dawley rats aged 8–10 weeks were used for the experiments. The studies were approved by the Animal Ethics Committee at Lund University.

Islet isolations, batch incubations, and islet perfusions. Rat islets from fed rats were isolated by standard collagenase digestion and subsequently handpicked under a stereo microscope. For culture overnight, the isolated islets were kept in RPMI-1640 medium, containing 11.1 mmol/l glucose, 10% FCS, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2.5 μg/ml fungizone, at 37°C in 95% air and 5% CO2. For secretion studies, batch (n = 8) or three islets for each condition were kept in HEPES balanced salt solution (HBSS; 125 mmol/l NaCl, 5.9 mmol/l KCl, 1.2 mmol/l MgCl2, 20 mmol/l HEPES, 1.3 mmol/l CaCl2, 0.1% BSA [pH 7.35]) containing 2.8 mmol/l glucose for 60 min in an incubator at 37°C. Then, three islets at a time were transferred to a multwell plate kept on ice and containing 200 μl per well of the same buffer but with the addition of the respective secretagogue and inhibitor. When all islets had been transferred, the plate was again placed in an incubator at 37°C; after 60 min, a sample from the buffer was removed for measurement of insulin by enzyme-linked immunosorbent assay (Mercodia, Uppsala, Sweden). In experiments with orlistat, the lipase inhibitor was also present during the 1-h preincubation at 2.8 mmol/l glucose.

Perfusion of islets was performed as previously described in detail (17). In brief, islets were preincubated in HBSS at 2.8 mmol/l glucose for 60 min. Then, batches of 20 islets were sandwiched between two layers of gel (Bio-Gel P4; Bio-Rad, Richmond, CA). The HBSS was warmed to 37°C. Islets were perfused at a flow rate of 0.5 ml/min, fractions were collected at 1-min intervals, and insulin was measured by enzyme-linked immunosorbent assay. For the secretion experiments, palmitate was dissolved in ethanol, after which a stoichiometric amount of NaOH was added. The ethanol was evaporated under N2, and the sodium palmitate was redissolved in H2O under heat and diluted as a 10-mmol/l stock solution in ice-cold 10% BSA (free fatty acid free). This stock solution was diluted 10 times in the HBSS at the time of the experiment. The free palmitate concentration, using this preparation, was estimated to be 5.6 mmol/l (18).

Lipolysis in islets and adipocytes. Batches of 100 islets were isolated and subsequently incubated at 37°C in 500 μl of HBSS, containing 2.8 or 16.7 mmol/l glucose with or without 200 μmol/l orlistat; in some experiments, 1 mmol/l 8-bromoadenosine-3′,5′-cyclic monophosphate (8-Brc-cAMP), a cAMP analog with enhanced stability, was included throughout. Incubations lasted for 3 h. Adipocytes were isolated from gonadal fat pads by collagenase digestion. Samples of the cell suspension were transferred to siliconized tubes containing prewarmed medium; final lipocit was 1–2%. After a 1-h incubation at 37°C with gentle shaking, cells were centrifuged through silicone oil to separate cells from the incubation medium. Glycerol released into the medium was measured as an index of lipolysis and was measured as described (19).

Enzyme-catalyzed lipolysis. The white adipose cell suspension was homogenized and islets were sonicated in buffer (0.25 mol/l sucrose, 1 mmol/l EDTA [pH 7.0], 1 mmol/l dithioerythritol, 20 μg/ml leupeptin, 20 μg/ml antipain, and 1 μg/ml pepstatin A) to allow assay of diglyceride lipase activity, as described elsewhere (20); the diacylglycerol analogue monooleoyl-2-O-monooleoylglycerol (MOMG) was used as substrate (21).

Glucose oxidation. For the secretion experiments, palmitate was dissolved in ethanol, after which a stoichiometric amount of NaOH was added. The ethanol was evaporated under N2, and the sodium palmitate was redissolved in H2O under heat and diluted as a 10-mmol/l stock solution in ice-cold 10% BSA (free fatty acid free). This stock solution was diluted 10 times in the HBSS at the time of the experiment. The free palmitate concentration, using this preparation, was estimated to be 5.6 mmol/l (18).
Diglyceride lipase activity in rat islets and adipocytes. To confirm that the effect of orlistat is due to inhibition of lipases, we assayed the diacylglycerol hydrolytic activity of extracted proteins from rat islets and adipocytes in the foregoing experiments. As shown in Fig. 3, diglyceride lipase activity was readily detected in islets incubated at either 2.8 or 16.7 mM in the absence or presence of 8-Br-cAMP. However, in the presence of 200 μmol/l orlistat, lipase activity, as measured against the synthetic diacylglycerol substrate MOME, was abolished. Similarly, diglyceride lipase activity was readily detected in protein extracts from the isolated rat adipocytes (Fig. 4); this same activity was significantly reduced by 71% (P < 0.01) in the presence of 200 μmol/l orlistat.

Insulin secretion in static incubations of rat islets. Next, we examined whether inhibition of lipases and consequently lipolysis would affect insulin secretion. As shown in Fig. 5A, a rise in glucose from 2.8 to 16.7 mM provoked a sixfold rise in insulin release from the islets. This increase was further potentiated fivefold by the addition of forskolin (Fig. 5B). However, addition of increasing concentrations of orlistat to the islets during the incubation resulted in a dose-dependent inhibition of both glucose-stimulated (Fig. 5A) and forskolin-potentiated insulin secretion (Fig. 5B). At the highest orlistat concentration used (200 μmol/l), release of insulin stimulated by 16.7 mM glucose alone was inhibited by 60% (P < 0.001); similarly, forskolin-potentiated insulin secretion was inhibited by 68% (P < 0.001).

Because we hypothesized that the inhibition by orlistat of glucose- and forskolin-stimulated insulin secretion is due to a block of mobilization of lipids from endogenous stores, provision of external lipids may serve this signaling role of the endogenous lipid(s) and thus overcome the inhibition of insulin secretion. To address this possibility, we performed a new set of static incubations in the presence of 1 mM palmitic acid (Fig. 6). Again, when orlistat was added to the islets, insulin secretion provoked by 16.7 mM glucose was markedly inhibited (−47%; P < 0.01); this inhibition was completely overcome by the addition of 1 mM palmitic acid.

Insulin secretion from perifused rat islets. Static incubations of islets fail to reveal dynamics of insulin release in terms of phasic secretion. Because lipids have been proposed to mediate K_{ATP}-independent glucose sensing (3), which is thought to drive second-phase insulin release, we examined the impact of orlistat on insulin secretion in a perifusion system. Thus, after perfusion of islets at nonstimulatory glucose (2.8 mM), the glucose concentration was raised to 16.7 mM (Fig. 7A). As a result, insulin secretion rose sevenfold, then followed a reduction in insulin release, subsequently followed by another, albeit slower, sustained rise in insulin secretion. At 40 min, glucose in the perfusate was reduced to 2.8 mM, which resulted in a fall in insulin secretion to baseline levels. At 50 min, 35 mM KCl was added to the perfusate, resulting in a sharp 20-fold increase in insulin secretion. Thus, the rat islets exhibited a clear biphasic secretion of insulin.

When orlistat was present in the perfusate throughout the experiment, an inhibition of GSIS was observed (Fig. 7A), thus reproducing our findings in static incubations of islets. The first phase of insulin secretion, defined as AUC from 14 min (insulin began to rise from basal levels) to the peak insulin value at 18 min, was not significantly altered by the presence of orlistat (−15%; Fig. 7B). However, the second phase of insulin secretion, defined as insulin release from 19 to 40 min, i.e., when glucose was lowered to 2.8 mM, was reduced by 25% (P < 0.05) in the presence of orlistat (Fig. 7B). KCl-induced insulin secretion was not significantly different in the two groups.

ATP levels in rat islets exposed to orlistat. If ATP: ADP ratios, the increase of which closes the K_{ATP} channel, are altered by orlistat, then it is less likely that the drug exerts its effects on insulin secretion via impaired formation of a lipid-derived signal critical for K_{ATP}-independent

**FIG. 1. Lipolysis in isolated rat islets.** Fresh isolated rat islets were cultured for 3 h at the indicated glucose concentrations, in the presence or absence of 1 mM 8-Br-cAMP and/or 200 μmol/l orlistat. Lipolysis was monitored by glycerol release; seven independent experiments were performed. *P < 0.05; **P < 0.01.

**FIG. 2. Lipolysis in primary rat adipocytes.** Lipolysis in primary adipocytes exposed to 2.5 μmol/l forskolin for 1 h, with or without 200 μmol/l orlistat, was monitored by glycerol release; four independent experiments were performed. ***P < 0.001 vs. forskolin.
glucose sensing. Instead, a general metabolic incapacitation may have occurred. To address this possibility, we measured islet levels of nucleotides and calculated ATP:ADP ratios. As shown in Fig. 8A, after a rise in glucose from 2.8 to 16.7 mmol/l, the ATP:ADP ratio increased significantly at 5 min; this increase was similar in islets incubated in the presence or absence of 200 μmol/l orlistat, demonstrating that the lipase inhibitor does not interfere with metabolically generated ATP.

**Glucose oxidation in rat islets exposed to orlistat.** To ensure further that the lipase inhibitor does not exert its negative effects on insulin secretion via an unspecific effect on metabolism, we examined oxidation of glucose in the presence and absence of orlistat. Indeed, we were able to demonstrate that HSL is expressed and active in β-cells (6). From immunoinhibition experiments, in which an antibody to HSL is used to block lipase activity (6), this was not a surprising finding, considering that it has long been recognized that β-cells store triglycerides and utilize lipids as fuel when ambient glucose is low (34). If it is assumed that generation of a lipid is involved in control of insulin secretion, then regulation of its level is a potential regulatory mechanism. Clearly, a lipase that mobilizes lipids from triglyceride stores and that is regulated by negative effects on insulin secretion, via an unspecific effect on metabolism, we examined oxidation of glucose in the presence and absence of orlistat. As expected, oxidation of the sugar increased fourfold at 16.7 mmol/l versus 2.8 mmol/l (Fig. 8B). Addition of orlistat, however, had no significant impact on glucose oxidation at either 2.8 or 16.7 mmol/l glucose.

**DISCUSSION**

Despite the widely embraced view that lipids play a role in the regulation of insulin secretion (25), little is known about the actual details of this involvement. A number of possibilities exist, one being that a lipid moiety in the β-cell arises as a result of glucose metabolism and participates in nutrient sensing (3,26). This is the long-chain acyl-CoA model, in which malonyl-CoA levels rise as a result of the cataplerotic exit of citrate from the tricarboxylic acid cycle, ultimately forming malonyl-CoA; this metabolite switches lipid metabolism from oxidation to esterification by virtue of its inhibition of the carnitine palmitoyl transferase I (27), the transporter of long-chain acyl-CoA into the mitochondrion for subsequent oxidation. Although there is a body of circumstantial evidence supporting this model (28,29), studies addressing its molecular mechanisms have both agreed (30,31) and disagreed (32,33) with how the model was initially put forth. Another possibility is that lipids provided from the circulation play an important role in insulin secretion, particularly during fasting, when the cellular lipid stores may be low (2).

An interesting new idea, however, has arisen from the observation that β-cells exhibit lipase activity (6). This was not a surprising finding, considering that it has long been recognized that β-cells store triglycerides and utilize lipids as fuel when ambient glucose is low (34). If it is assumed that generation of a lipid is involved in control of insulin secretion, then regulation of its level is a potential regulatory mechanism. Clearly, a lipase that mobilizes lipids from triglyceride stores and that is regulated by intracellular second messengers is an attractive candidate for such a role. These characteristics apply to HSL (7). In adipocytes, this lipase is activated by reversible phosphorylation of serine residues by PKA (8). Thus, triglyceride hydrolysis is stimulated by a rise in intracellular cAMP. Indeed, we were able to demonstrate that HSL is expressed and active in β-cells (6). From immunoinhibition experiments, in which an antibody to HSL is used to block diglyceride lipase activity (6,24), it was deduced that ~25% of such activity in INS-1 cells is accounted for by HSL. Moreover, chronic exposure to high glucose induces expression and activity of HSL in INS-1 cells and rat islets and, in parallel, stimulates lipolysis, as determined in INS-1 cells (24). Recently, it was shown that the incretin hormone and cAMP-raising agent GLP-1 stimulates an intracellular acidification of HIT-T15 cells and an efflux of fatty acids (14); these events were attributed to a stimulation of lipolysis, presumably accounted for by HSL, given that cAMP mediates the effect observed. Furthermore, orlistat was demonstrated to inhibit forskolin- and GLP-1–stimulated insulin secretion, while being less effective in GSIS (14), and yet another antilipolytic agent, 3,5-dimethylpyrazole, also inhibits insulin secretion induced by glucose, cAMP-raising agents, and α-ketoisocaproic acid (10).

Although these observations support a role for lipases generating lipid signals in GSIS, studies in HSL null mice have been less clarifying. In one line of HSL null mice,
GSIS is impaired in vivo and in vitro (11). However, another line of HSL null mice, created in our laboratory, fails to exhibit an impairment of insulin secretion (12). The reason for this apparent discrepancy is unclear but could be explained by genetic redundancy of other lipid-hydrolyzing enzymes. In fact, the existence of such lipases is highly likely, given our previous observation that lipase activity in vitro in INS-1 cells is only partially blocked by HSL-neutralizing antibodies (6,24). Instead, the outstanding finding concerning metabolism in our line of HSL null mice is reduced insulin sensitivity (12); in vivo, the mice appropriately adapt to insulin resistance by hypersecretion of insulin, in part accounted for by islet hypertrophy.

The experiments in the present study were carried out in an attempt to confirm that lipases play a role in insulin secretion. To this end, orlistat, an inhibitor of a wide spectrum of lipases, was used to inhibit all lipases in primary β-cells. Orlistat is highly lipophilic, is insoluble in water, and binds avidly to proteins. Despite these features, it is known that orlistat at high concentrations traverses cellular membranes (35) and blocks intracellular lipases (14,36). Orlistat binds irreversibly to the catalytic site of lipases and has been shown to inhibit HSL (13). Here, we were able to show that acute exposure to glucose stimulated lipolysis in rat islets. This was further potentiated by 8-Br-cAMP, suggesting that HSL plays a role in these processes, because the nucleotide will activate HSL via PKA (7). Addition of orlistat to the islets blocked lipolysis as well as diglyceride lipase activity, strongly indicating that the efflux of glycerol from the islets emanated from lipolysis. This conclusion assumes that glycerol is not generated from other sources, because an enzyme, such as glycerol-3-phosphatase in yeast, has not been reported in β-cells. However, that inhibition of diglyceride lipase activity by orlistat was more extensive than the abrogation of glycerol efflux could indicate that glyceroneogenesis in fact occurs in islets. This is difficult to reconcile with the lacking activity of, for example, phosphoenolpyruvate carboxykinase in islets (37), and therefore warrants further study. Alternatively, the residual lipase activity after orlistat treatment may be sufficient to generate glycerol.

Nevertheless, experiments in adipocytes yielded similar results, showing that the effect of orlistat is not restricted to one cell type. In static incubations of rat islets, orlistat dose dependently inhibited GSIS; the effect on forskolin-induced insulin secretion was also marked. That this inhibition, in fact, stems from a block of lipid mobilization is supported by the observation that exogenous lipid, in the form of palmitic acid, recovered insulin secretion in the presence of orlistat. To exclude that the effect of orlistat was due to nonspecific toxic action of the lipase, we also determined the ATP:ADP ratio in the islets and oxidation of glucose. We found that the increase in the ATP:ADP ratio provoked by a rise in glucose as well as oxidation of the sugar was unaffected by orlistat, yet insulin secretion was clearly inhibited. This indicates that the insulinoostatic effect of orlistat cannot be attributed to
inhibition of glucose metabolism with impaired production of ATP or that a more general incapacitation of β-cell function has occurred.

It is interesting that perifusion of islets revealed that mainly the second phase of insulin secretion was inhibited by orlistat. A more pronounced effect on the second phase of insulin secretion than on the first phase suggests that $K_{\text{ATP}}$-independent glucose sensing is impaired. This fits well with the presumed action of orlistat in β-cells, namely impaired mobilization of lipids from stored acylglycerides. The implication of our findings is therefore that a lipid generated by acylglyceride hydrolysis plays a critical role in insulin secretion. At this point, attention must be turned toward the identity of these lipid factors and the lipase(s) by which they are generated. Whether HSL is one of these or perhaps the only one remains to be determined.

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