

Fatty acids and insulin secretion

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It has long been recognized that acute elevation of non-esterified fatty acids (NEFA) stimulates insulin secretion to a moderate extent both *in vitro* and *in vivo*. The effects of longer-term exposure to elevated fatty acids have, however, been investigated only recently. Our own studies in the rat have documented the time dependence of NEFA effects, with inhibition of glucose-induced insulin secretion being apparent after 6–24 h *in vivo* exposure to Intralipid or *in vitro* exposure to palmitate, oleate and octanoate. Evidence indicates that the inhibitory effects are coupled to fatty acid oxidation in B-cells, with ensuing reduction in glucose oxidation, in parallel with diminished activity of the pyruvate dehydrogenase enzyme. These findings were essentially confirmed in human pancreatic islets. In the *db/db* mouse, a model of type 2 diabetes with obesity, evidence was obtained for elevated NEFA playing a significant role in decreased glucose-induced insulin secretion. Evidence also indicates that elevated NEFA inhibit insulin biosynthesis and increase the proinsulin : insulin ratio of secretion. Results on experimentally induced elevations of NEFA in non-diabetic and diabetic humans are thus far inconclusive. Further studies are needed to ascertain the impact of elevated NEFA on insulin secretion in clinical settings.

Islets of Langerhans: Diabetes mellitus: Fatty acids: Insulin secretion

Introduction

The B-cell is exquisitely sensitive to glucose. Even small alterations in blood glucose normally evoke large effects on insulin secretion. This sensitivity is coupled to metabolism of glucose in the B-cell. Given that metabolic signals are crucial for insulin secretion, it is natural to ask whether nutrients other than glucose are important for regulation of B-cell function. Fatty acids are of special interest in this regard as (i) they make up a major part of B-cell fuel consumption (Berne, 1975; Malaisse *et al.* 1985); (ii) non-esterified fatty acids (NEFA) are elevated in many diabetic subjects, particularly in those with non-insulin-dependent diabetes mellitus (NIDDM) and obesity; (iii) fatty acids induce insulin resistance in many organs.

Earlier studies have shown that NEFA stimulate insulin secretion at both normal and elevated glucose (Malaisse & Malaisse-Lagae 1968; Pelkonen *et al.* 1968; Crespin *et al.* 1969). However, the stimulatory effects are moderate and somewhat disproportionate to the major contribution of fatty acids to B-cell total metabolism. These studies therefore assigned only a minor role for fatty acids in the overall regulation of insulin secretion.

Earlier studies investigated short-term effects of fatty acids on insulin secretion; longer-term effects of fatty acids on B-cell function were not investigated in any detail. In diabetes, elevated fatty acids may be present for

years and even decades. Clearly, long-term effects of fatty acids would be dominant in many diabetic patients. For these reasons we began to test for such effects on B-cell function.

Demonstration of longer-term effects of fatty acids

Our first study (Sako & Grill, 1990a) was performed in normal rats. The rats received a continuous infusion of a fat emulsion, Intralipid, for 3, 6 or 48 h. The Intralipid infusion raised the levels of NEFA threefold. Insulin responses to glucose were measured in the isolated perfused pancreas. A 3 h infusion increased the subsequently tested insulin response to glucose. This stimulatory effect was lost after 6 h of Intralipid infusion. Extending the infusion to 48 h led to a 50 % inhibition of glucose-induced insulin secretion. These inhibitory effects in the perfused pancreas were also found in isolated islets from the 48 h Intralipid-infused rats.

To investigate the mechanisms behind the inhibitory effect on glucose-induced insulin secretion, we measured glucose oxidation in isolated islets from 48 h Intralipid-infused rats. A high glucose concentration *in vitro* markedly increases islet glucose oxidation. Previous Intralipid attenuated this stimulatory effect of high glucose. To test whether the inhibitory effect of previous Intralipid was

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coupled to fatty acid oxidation, we tested for the effects of sodium 2-[6-(4-chlorophenoxy)-hexyl]oxirane-2-carboxylate, Etomoxir. This compound inhibits fatty-acid oxidation by inhibiting the carnithine-palmitoyl transferase I (CPT-I) enzyme, the activity of which is necessary for the transport of long-chain fatty acid residues into the mitochondria where oxidation takes place. Adding Etomoxir *in vitro* to islets of Intralipid-infused rats significantly ameliorated both glucose oxidation and glucose-induced insulin secretion. These results indicate that the inhibition of glucose metabolism by long-term exposure to elevated fatty acids is linked to fatty acid oxidation.

Tissue culture studies were performed to investigate in more detail how fatty acids induce their inhibitory effects on B-cell function. Islets from normal rats were exposed to selected fatty acids: palmitate, oleate or octanoate during 6, 24 and 48 h tissue culture conditions. The results confirmed a time-dependency for the inhibitory effects as was first demonstrated with Intralipid *in vivo* (Zhou & Grill, 1994). *In vitro*, a 24 h period of exposure to fatty acids was needed to document an inhibitory effect on glucose-induced insulin secretion (Fig. 1). In contrast, a moderate stimulatory effect on basal insulin secretion was observed at all times after fatty acid exposure (Fig. 1). The inhibitory effects were reversible within 24 h, indicating that the effects observed were not due to unspecific toxicity.

The tissue-culture study showed an additional inhibitory effect on glucose regulation of B-cell function, namely on glucose-induced insulin and total protein biosynthesis. Such an effect has also been confirmed by Bollheimer *et al.* (1998). An effect on insulin biosynthesis may explain the lowered insulin content that was found in fatty acid-exposed

islets, especially as no effects on intracellular breakdown of insulin could be found in the Bollheimer study.

Role of PDH and PDH kinase activities for fatty acid oxidation

Which step(s) of glucose metabolism are inhibited by fatty acid oxidation? Our attention turned to the pyruvate dehydrogenase (PDH) enzyme complex. The PDH enzyme complex is a key regulator of glucose entry into the Krebs cycle. Activity of the PDH enzyme complex is allosterically regulated by substrates and products of the enzymatic reactions. Activity is also regulated by phosphorylation and dephosphorylation. Phosphorylation inactivates PDH activity and is achieved through the activity of PDH kinase, whereas activation occurs through dephosphorylation by phosphatases.

In rat pancreatic islets we found that a 48 h period of fatty acid exposure *in vitro* led to a decrease in the percentage of PDH being in the active, unphosphorylated form (Zhou & Grill, 1995b). Concomitantly, we observed that long-term exposure to fatty acids increased PDH kinase activity in islet mitochondria.

The PDH kinase enzyme is known to be strongly associated with the PDH enzyme complex. More recent studies in liver cells have shown that PDH kinase also exists in a non-PDH-bound form (Priestman *et al.* 1994). The activity of 'free' PDH kinase was demonstrated to increase markedly as a result of, for instance, longer-term exposure to fatty acids. Against this background, we proceeded to measure separately 'free' and PDH-bound PDH kinase from mitochondria of the rat pancreatic islets. Activity measurements

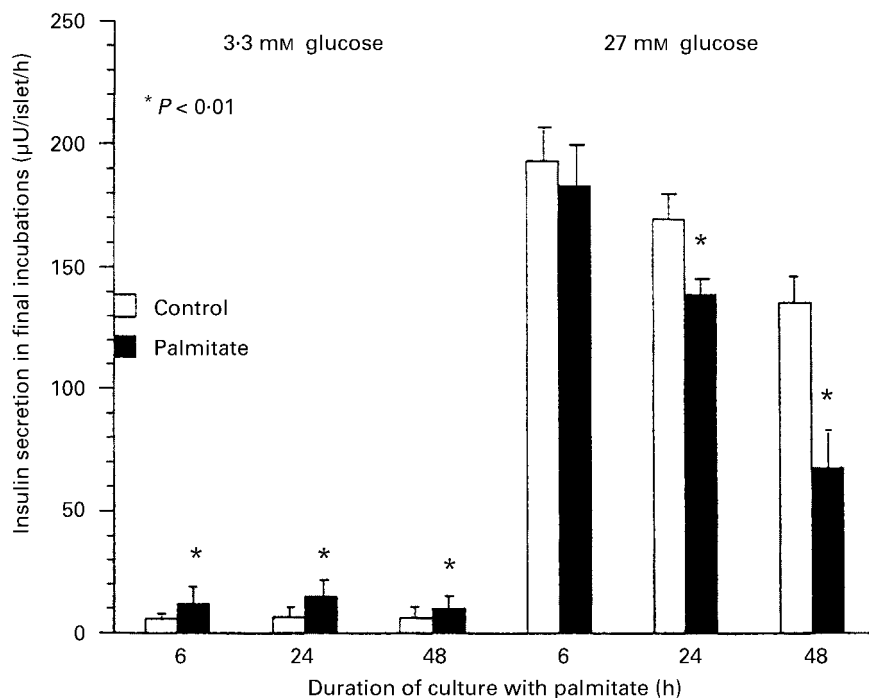


Fig. 1. Time dependency of palmitate exposure for inhibition of glucose-induced insulin secretion. Results derived from Zhou & Grill (1994) and reproduced with permission of the *Journal of Clinical Investigation*.

after such separation showed that long-term fatty acid exposure preferentially increased the activity of islet 'free' PDH kinase.

Fasting, elevated NEFA and PDH and PDH kinase activities

Fasting and starvation lead to elevated fatty acids, increased fatty acid oxidation, and insulin resistance. Evidence exists that induction of insulin resistance is, at least in part, caused by the operation of a glucose fatty acid cycle, and that increased PDH kinase activity constitutes an important part of the insulin resistance mechanisms. Fasting and starvation are also associated with a decreased insulin response to glucose (Malaisse *et al.* 1967). Against this background we tested the importance of elevated fatty acids during fasting for the insulin response to glucose and, in particular, for effects on PDH and PDH kinase activities. Our results in 48-h-starved rats showed an expected reduced insulin response to glucose (Zhou *et al.* 1996c). We also found a reduced oxidation of [¹⁴C]glucose in the pancreatic islets from the starved rats. The ratio of glucose oxidation (oxidation of [¹⁴C]glucose) to glucose utilization (production of tritiated water from 5-H³-labelled glucose) was decreased in islets from fasted rats. This indicates that the aerobic metabolism of glucose was affected, rather than the anaerobic metabolism. We also found that the percentage of active PDH was reduced, whereas the activity of PDH kinase was increased. These results supported the hypothesis that elevated fatty acids during fasting would affect B-cell secretion and metabolism by mechanisms similar to those operating during the *in vivo* and *in vitro* procedures employed in the experiments presented above. As a further support for this notion, the CPT-I blocking agent Etomoxir partially restored an insulin response to glucose in islets from starved rats, and also increased the oxidation of glucose in the same islets.

Seemingly in contrast to our results, Stein *et al.* (1996) reported that the presence of extracellular fatty acids was essential for insulin secretion in the fasted but not in the fed rat, a finding also reported in man (Dobbins *et al.* 1998). The reasons for the discrepancies have not been clarified. It seems possible that necessity for fatty acids could pertain to lower concentrations and/or shorter exposure times than those employed in our studies. Indeed, in 54-h-fasted humans we find that administration of Intralipid potentiates glucose-induced insulin secretion initially, but not during prolonged lipid infusion (unpublished results).

Other metabolic effects of long-term elevated NEFA

In an insulinoma cell line it has been reported that reduction of glucose oxidation due to fatty acids is not sufficient to explain the inhibitory effects on insulin secretion, when calculated from expected production rates of ATP (Liang *et al.* 1997). It is possible that long-term elevated fatty acids could, to some extent, induce uncoupling of mitochondrial ATP production in B-cells and that the decreased efficiency of metabolism would negatively affect insulin responses to glucose. This notion is supported by a recent publication (Carlsson *et al.* 1999).

Triglyceride stores

Others (Lee *et al.* 1994), as well as ourselves (Zhou *et al.* 1996b) have found that long-term elevated NEFA lead to increased islet stores of triglycerides. Such stores are a prerequisite for demonstrating *in vitro* negative effects of previously elevated NEFA. It may be questioned whether the negative effects that we have observed are explained by markedly enhanced beta oxidation *in vitro*, such enhancement being secondary to massive intra-islet lipolysis at the time of functional testing. At least one observation speaks against the notion that the inhibitory effects are due to, and limited to, such artificial conditions. Blocking CPT-I activity by Etomoxir, we found that triglyceride breakdown is markedly retarded, indicating that major lipolysis from intra-islet stores does not occur in the presence of the CPT-I inhibitor (Zhou *et al.* 1996b). The CPT-I enzyme activity is needed for transport over the outer mitochondrial membrane of long-chain fatty acid residues. Medium-chain fatty acids, such as octanoate, do not need this uptake mechanism. With Etomoxir present, exogenous octanoate would in principle be the only fatty acid taken up by mitochondria. Under such experimental conditions we found that previous exposure to palmitate did not change the oxidation rate of octanoate (production of ¹⁴CO₂ from ¹⁴C-labelled octanoate). However, following long-term exposure to palmitate, the acute administration of octanoate decreased islet glucose metabolism. These observations are compatible with the build-up of negative 'sensing' of fatty acid oxidation following increasing times of exposure to fatty acids. Such negative sensing could include a time-dependent, induced increase in PDH kinase activity.

It has been proposed that accumulated triacylglycerols may be potentially toxic to B-cells, causing cellular depletion and fibrosis (Shimabukuro *et al.* 1998). Evidence for this notion was obtained in diabetic Zucker *fa/fa* rats. Because these rats harbour a leptin receptor mutation, they accumulate 50- to 100-fold more triacylglycerols in their B-cells than do normal rats. It remains to be demonstrated whether only a moderate increase in B-cell triacylglycerols would exert toxic effects.

Effects of NEFA on B-cell function in animal models of type 2 diabetes

The *db/db* mouse is hyperphagic because of a defect in the hypothalamic leptin receptor. When the *db* gene is present on the C57BL/KsJ background, the animal develops diabetes early in life (Berglund *et al.* 1978). After a 3–6-month period of hyperinsulinaemia and adiposity, insulin secretion successively diminishes. The phenotypic traits of the *db/db* mouse bear resemblance to the course of diabetes in obese NIDDM patients. We therefore investigated the influence of elevated NEFA on B-cell function in this animal model (Zhou *et al.* 1996a). Three-month-old *db/db* mice were, as expected, obese, hyperglycaemic and hyperinsulinaemic. Their levels of NEFA were significantly elevated compared with their lean *db/+* littermates. Insulin responses to glucose were greatly reduced in islets from *db/db* mice. Oxidation of ¹⁴C was reduced, both in absolute terms and in relation to glucose utilization. Also the percentage of the active

(unphosphorylated) form of PDH was decreased. Exposure to the CPT-I inhibitor Etomoxir significantly reversed these abnormalities; however Etomoxir did not achieve normalization. Taken together, these results indicate that long-term elevated NEFA with resulting increased oxidation of NEFA inhibits B-cell function in the *db/db* mouse to a significant extent, and that a decrease in PDH activity is part of this effect. The results also highlight the fact that factors other than elevated NEFA exert negative influences on B-cell function (see below).

Effects of long-term elevated NEFA on human islets

To investigate whether our observations in rats and mice were applicable to human B-cells, we studied the influence of elevated fatty acids on human islets *in vitro*. Human islets were obtained through the Beta Cell Transplant Unit in Brussels. Fatty acids (palmitate or oleate), when present during 48 h tissue culture conditions, inhibited the subsequently tested glucose-induced insulin secretion (Zhou & Grill, 1995a). We also observed an inhibitory effect on PDH activity in the human islets. Moreover, as in rat islets the glucose effect on protein synthesis (proinsulin and total protein) was attenuated by previous exposure to palmitate.

A further effect of fatty acids in cultured human islets was an enhanced proinsulin : insulin ratio of secretion (Björklund & Grill, 1999). This effect was noted after 48 h exposure to palmitate. Culture at high glucose levels enhanced proinsulin : insulin ratios; the effect of palmitate was, however, additive to the effect of high glucose levels (Table 1). The effect of palmitate could be secondary to delayed processing of proinsulin, as documented in a rat insulinoma cell line (Furukawa *et al.* 1999).

Role of elevated NEFA for B-cell function under diabetic conditions

Several observations support a role of elevated NEFA in decreased insulin responses to glucose in animal models of diabetes, including our results in the *db/db* mouse. Others have found that a high-fat diet fed to diabetic mice inhibits B-cell function (Capito *et al.* 1992). Furthermore, elevated NEFA and increased islet triglyceride stores were found to precede diabetes in *fa/fa* rats (Lee *et al.* 1994).

In human islets we found that long-term elevated NEFA exerted negative effects in addition to those exerted by prolonged exposure to a high glucose concentration (Fig. 2). Combined effects of elevated NEFA and hyperglycaemia were also observed in a rat model of hyperglycaemia through glucose infusions (Sako & Grill, 1990a). In the latter experiments, hyperglycaemia achieved through glucose infusions markedly attenuated glucose-induced insulin secretion; however the co-infusion of Intralipid decreased glucose-induced insulin secretion even further.

It is thus clear that elevated NEFA is not the only metabolic abnormality that can compromise insulin secretion in diabetes. We have previously demonstrated the importance of intense or excessive B-cell stimulation for B-cell insensitivity to glucose after prolonged high

Table 1. Effects of a 48 h culture with palmitate on the proinsulin/insulin ratio of secretion

Culture conditions	Proinsulin : insulin ratio (%)
Glucose 5.5 mM	2.2 ± 0.4
Glucose 5.5 mM + palmitate	5.1 ± 0.9*
Glucose 27 mM	10.8 ± 1.2†
Glucose 27 mM + palmitate	13.9 ± 2.8*

Human pancreatic islets were cultured in RPMI with 0.2 mM palmitate and 5.5 or 27 mM glucose. Secretion was measured post-culture in 60 min incubations at 3.3 mM glucose without added fatty acids. Mean ± SE of nine separate experiments.

* $P < 0.05$ for effect of palmitate, † for effects of glucose.

Results derived from Björklund & Grill, 1999 and published with permission of the American Diabetes Association.

glucose exposure (Sako & Grill, 1990b). Another factor of importance can be referred to as 'glucotoxicity' *per se*, possibly linked to the formation of advanced glycosylation end products (Tajiri *et al.* 1997).

Returning to the influence of elevated NEFA, we would like to stress the potential importance of the negative effects on protein synthesis that we have observed (Zhou & Grill, 1994, 1995a). In the light of increased demands for insulin biosynthesis during conditions of hyperglycaemia and insulin resistance, any inhibitory effect on insulin biosynthesis seems inappropriate and potentially deleterious.

Different effects of different fatty acids?

Taken together, the results summarized in this paper indicate that saturated (palmitate), monounsaturated (oleate), and polyunsaturated fatty acids (predominant in

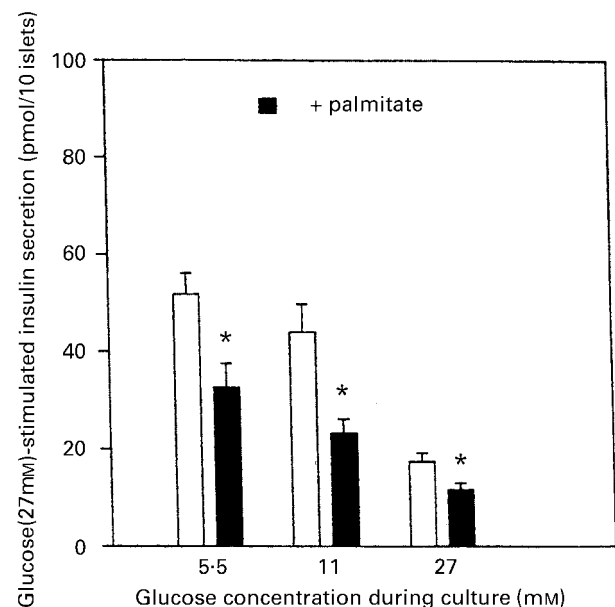


Fig. 2. Combined negative effects of glucose and fatty acids on glucose-induced insulin secretion from human pancreatic islets. Islets were cultured for 48 h at different glucose concentrations with or without palmitate. The insulin responses to 27 mM glucose were measured subsequent to the culture period. *, $P < 0.05$ for fatty acid effect. Results are derived from Zhou & Grill (1995a) and reproduced with permission of the *Journal of Clinical Endocrinology and Metabolism*.

Intralipid) exert inhibitory effects on glucose-induced insulin secretion. This does not exclude certain differences in the impact of individual fatty acids on B-cell function. Indeed, long-chain saturated fatty acids appear to stimulate insulin release more potently than unsaturated ones (Stein *et al.* 1997; Warnotte *et al.* 1999). Differences in effects between fatty acids have also been noted with regard to inhibitory effects on pre-proinsulin gene expression (Ritz-Laser *et al.* 1999); induction of immediate early-response genes (Roche *et al.* 1999); and inhibition of glucose oxidation (Alstrup *et al.* 1999). However, evidence so far does not allow the conclusion that certain fatty acids exert more negative effects than others on B-cell function.

Long-term effects of elevated NEFA on B-cell function in man

A time-dependent negative NEFA effect was reported in one study performed in non-diabetic humans (Paolisso *et al.* 1995). In another study, however, only stimulatory effects of elevated NEFA were demonstrated (Boden *et al.* 1995). In a third study (Carpentier *et al.* 1999), a 48 h infusion of Intralipid did not affect glucose-induced insulin secretion; however, it was argued that secretion should normally have been increased in response to the concomitant induction of insulin resistance. Recently we tested the effect of acutely decreasing elevated NEFA in 22 type-2 diabetic subjects by a nicotinic acid derivative, Acipimox. The administration of Acipimox 60 min before a hyperglycaemic clamp enhanced glucose-induced insulin secretion by about 20 % (unpublished results). These results could suggest that glucose-induced insulin secretion is to some extent tonically suppressed by elevated NEFA in human type-2 diabetes.

The well known insulin-resistance effect of elevated fatty acids makes it difficult to interpret *in vivo* insulin data. The influence of fatty acids is reminiscent of the effects of glucocorticoids which enhance insulin secretion secondarily to their resistance effect, but *in vitro* these hormones clearly inhibit insulin secretion (Billaudel & Sutter, 1979). Although these discrepant effects of glucocorticoids have been known for many years, it has been difficult to determine to which extent a negative effect will hamper the resistance-induced enhancement of insulin secretion. Similar difficulties are encountered in elucidating the importance of fatty acids for insulin secretion in diabetes.

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