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Chronic effect of fatty acids on insulin release is not through the alteration of glucose metabolism in a pancreatic beta-cell line (βHC9)

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Summary Hyperinsulinaemia in the fasting state and a blunted insulin secretory response to acute glucose stimulation are commonly observed in obesity associated non-insulin-dependent diabetes mellitus. Hyperlipidaemia is a hallmark of obesity and may play a role in the pathogenesis of this beta-cell dysfunction because glucose metabolism in pancreatic beta cells may be altered by the increased lipid load. We tested this hypothesis by assessing the chronic effect of oleic acid on glucose metabolism and its relationship with glucose-induced insulin release in βHC9 cells in tissue culture. Our results show: (1) A 4-day treatment with oleic acid caused an enhancement of insulin release at 0–5 mmol/l glucose concentrations while a significant decrease in insulin release occurred when the glucose level was greater than 15 nmol/l; (2) Hexokinase activity was increased and a corresponding left shift of the dose-dependency curve of glucose usage was observed associated with inhibition of glucose oxidation in oleic acid treated βHC9 cells, yet the presumed glucose-related ATP generation did not parallel the change in insulin release due to glucose; (3) The rate of cellular respiration was markedly increased in oleic acid treated βHC9 cells both in the absence of glucose and at all glucose concentrations tested. This enhanced oxidative metabolism may explain the increased insulin release at a low glucose level but is clearly dissociated from the blunted insulin secretion at high glucose concentrations. We conclude that a reduction of oxidative metabolism in pancreatic beta cells is unlikely to be the cause of the dramatic effect that high levels of non-esterified fatty acids have on glucose-induced insulin release. [Diabetologia (1997) 40: 1018–1027]

Keywords Islet of Langerhans, lipotoxicity, βHC9 cells, glucose metabolism

Obese patients have a relatively high risk for developing non-insulin-dependent diabetes mellitus (NIDDM). Insulin resistance and pancreatic islet dysfunction contribute to the development of the disease. A distortion of insulin release when related to prevailing blood glucose is characteristic of the islet dysfunction in these patients. In the fasting state, when the blood glucose level is relatively low, the insulin level is disproportionately high in these patients compared with normal subjects. However, when the blood glucose level is high, (e.g. postprandially), the insulin level remains abnormally low. The molecular basis of this islet dysfunction in obesity associated NIDDM patients remains unclear. Among many proposed possibilities, hyperlipidaemia which is commonly found in obese patients may constitute a critical pathological factor in the genesis of beta-cell dysfunction [1, 2].

Acute effects of non-esterified fatty acids (NEFA) on insulin release have been studied for more than two decades. NEFA may serve as fuel for pancreatic beta cells [3] and when islets are acutely exposed to

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Abbreviations: NIDDM, Non-insulin-dependent diabetes mellitus; NEFA, non-esterified fatty acids; DMEM, Dulbecco's modified Eagle's medium; BSA, bovine serum albumin.

NEFA, insulin release increases significantly provided "permissive" levels of glucose are present [3, 4, 5]. This stimulatory effect of NEFA has been attributed to fatty acid oxidation or to activation of protein kinase C by long chain acyl-CoA [1]. Chronic effects of NEFA on insulin release have been explored only recently and several studies revealed a complex picture. Long-term exposure of islets to NEFA resulted in high insulin release at relatively low glucose concentrations between 2 and 5 mmol/l [6], whereas insulin release was significantly reduced when islets pretreated with NEFA were stimulated by 16 mmol/l glucose [7]. It has been proposed that these changes in insulin response may be the consequences of an impairment in islet glucose metabolism. The most pronounced effects of NEFA on islet glucose metabolism described so far are an apparent increase of hexokinase activity [6] and indications of a decreased activity of pyruvate dehydrogenase [7]. These enzymes catalyze regulatory steps in glycolysis and mitochondrial oxidation. Changes of these two enzymes could greatly alter glucose metabolism in pancreatic beta cells and thus explain the observed defect in the glucose regulated insulin release.

The present study was performed to test this hypothesis using βHC9 cells. This particular pancreatic beta-cell line possesses normal characteristics of glucose metabolism and insulin release due to glucose [8]. Thus it offers an ideal cell model for assessing chronic effects of NEFA on glucose metabolism of pancreatic beta cells in culture directly without being influenced by homeostasis in the intact body. We analysed the kinetics of glucose metabolism in βHC cells treated for 4 days with 2 mmol/l oleic acid, measured their oxygen consumption at different glucose concentrations, and estimated the rate of ATP generation of these cells when exposed to glucose. We found that in NEFA treated βHC9 cells, the presumed glucose-related ATP generation was practically unchanged when compared to controls, but that the total cell oxygen consumption and therefore total ATP generation were markedly increased. Deficits in energy metabolism and ATP generation in these cells cannot be used, therefore, as an explanation for the changes in glucose-induced insulin release. We conclude that alterations in oxidative glucose metabolism are not the major cause of beta-cell dysfunction resulting from so-called "lipotoxicity" and postulate that other factors related to augmented lipid metabolism must be involved.

Materials and methods

General procedures. The βHC9 cell line was obtained from Dr. D. Hanahan [9] and maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum and 25 mmol/l glucose. To test the chronic effect of oleic acid, cells were fed with DMEM containing 2% bovine serum albumin (BSA) and oleic acid (Sodium salt, Sigma Chemical, St. Louis, Mo., USA) at concentrations of 1 or 2 mmol/l for different time periods as indicated in the Results. The DNA content in cells was determined by a diphenylamine method [10] using calf thymus DNA (Sigma Chemical) as standard. The cellular protein content was measured by a Coomassie Blue binding assay (Bio-Rad, Hercules, Calif., USA). Glucose stimulated insulin release was studied with static incubations of cells and a radioimmunoassay was utilized to measure insulin content in the incubation buffer [11].

Determination of glucokinase and hexokinase. The activities of glucokinase and hexokinase were measured as described earlier [12]. Briefly, cell homogenates were split equally into two parts. One part was used to determine the activities of glucokinase and hexokinase in the total cell homogenate and the other part was centrifuged and the supernatant portion was then used to quantitate enzyme activity in the cytoplasm. The enzyme assay was performed at 30 °C using a NADP-coupled fluorometric method. A factor of 2 was used to correct for the effect of 6-phosphogluconate dehydrogenase contained in the sample, since βHC cells contained enough enzyme to convert 6-phosphogluconate quantitatively to ribulose-5-phosphate [12]. The maximum velocity (V_{max}) and Michaelis-Menten constant (K_m) of these two enzymes were calculated by Eadie-Scatchard plots and the hexokinase activity was separated from glucokinase activity according to the method of Spears et al [13]. The enzymatic activity was corrected for temperature by using a $Q_{10} = 2.0$ for estimating glucose phosphorylation at 37 °C [14].

Glucose usage. Glucose usage was measured using a radiometric method [8]. Cells were first seeded in 12-well plates 2 days before the oleic acid treatment was begun. The determination of 5-[^{3}H]glucose usage was started after cells were preincubated with modified Hanks' buffer (pH 7.4) for 60 min in the absence of glucose at 37 °C in a shaking water bath. The preincubation solution was replaced by the assay reagent containing different glucose concentrations (0.06 to 32 mmol/l) with 4 μCi of 5- [^{3}H]glucose present and the incubation was continued for another hour. The reaction was stopped by adding 40 μl of 1 N HCl. A 100 μl aliquot was transferred to a diffusion tube which was then incubated at 34 °C overnight to separate ^3H_2O from the labelled glucose. After corrections for incomplete equilibration of the ^3H_2O and of the blank of water soluble volatile radioactive material in the tritiated glucose stock solution, glucose usage was calculated using the method of Ashcroft et al [15].

Determination of lactate production. Cells were seeded in 60-mm dishes 2 days before the oleic acid treatment was begun. Then, these cells were incubated with modified Hank's buffer containing different glucose concentrations (0.1–32 mmol/l) for 60 min. Next, perchloric acid was added at a final concentration of 0.5 N to denature the cells. The cleared perchloric acid extract was neutralized by KOH and the combined lactate content of cells and incubation medium was determined by an enzymatic fluorometric method [16].

Glucose oxidation. Cells were first seeded in 25 cm^2 flasks and cultured for 48 h before oleic acid treatment. Glucose oxidation was measured using a radiometric method [8]. In brief, cells were incubated with modified Hank's buffer containing different glucose concentrations (0.06–32 mmol/l) and 3 μCi of [U-^{14}C] glucose. After 60 min incubation, 400 μl of 1 N HCl was injected into the flask sealed by a rubber stopper and

200 μl of Hyamine was added to impregnate a filter paper that was present in centre wells of the flasks. After 2 h shaking, the filter paper together with the centre well was counted for $^{14}CO_2$ generated by the oxidation of glucose.

Oleic acid oxidation. Oleic acid oxidation in βHC9 cells was determined using [1–^{14}C] oleic acid. Cells were first seeded in 25-cm^2 flasks and cultured for 48 h before the oleic acid treatment. After 4 days' culture in the presence or absence of 2 mmol/l oleic acid, cells were incubated with modified Hank's buffer containing different glucose concentrations (0.06–32 mmol/l) and 4 μmol/l of [1–^{14}C] oleic acid (100000 dpm/nmol). After 60 min incubation, 400 μl of 1 N HCl was injected into the flask, sealed by a rubber stopper and 200 μl of Hyamine was added to impregnate a filter paper that was present in the centre wells of the flasks. After 2 h shaking, the filter paper together with the centre well was counted for $^{14}CO_2$ generated by the oxidation of oleic acid.

Cell respiration. Oxygen consumption of βHC9 cells was measured by a Clark electrode in a water-jacketed glass reaction vessel at 37 °C as described previously [8]. Cell suspensions were added to the reaction buffer in the absence of glucose. After 3 min of observing the basal oxygen consumption, glucose was injected into the sealed vessel through a capillary to reach a final concentration ranging from 1 to 30 mmol/l The oxygen uptake was then recorded for another 15 min.

Statistical analysis. All the data presented here are the mean ± SEM. Student's *t*-test was applied in the statistical analysis.

Results

The chronic effect of oleic acid on glucose-induced insulin release from βHC9 cells. To study the chronic effect of NEFA on βHC9 cells, we first examined glucose-induced insulin release from βHC9 cells cultured in the presence of oleic acid. After exposing βHC9 cells to 2 mmol/l oleic acid for 4 days, there was a significant change in the dose-response curve of insulin release due to glucose (Fig. 1). In the absence of glucose and also at glucose levels lower than 5 mmol/l, insulin release from oleic acid-treated cells was significantly higher than that in controls that did not receive oleic acid. However, when the glucose concentration in the buffer was higher than 15 mmol/l, the insulin release response was reduced compared with that in the controls. This effect of oleic acid was time-dependent, since in βHC9 cells treated with 2 mmol/l oleic acid for only 2 days, insulin release at 1–5 mmol/l glucose was not significantly increased and the glucose-stimulated insulin release was reduced only when 30 mmol/l glucose was present in the buffer (compare Fig. 1A and 1B). These alterations in βHC cell function are consistent with previous observations in cultured pancreatic islets [6, 7]. They also resemble the insulin release patterns that are observed in NIDDM associated with obesity. The results underscore the usefulness of using βHC9 cells in culture to study the mechanisms of chronic NEFA overexposure on insulin release.

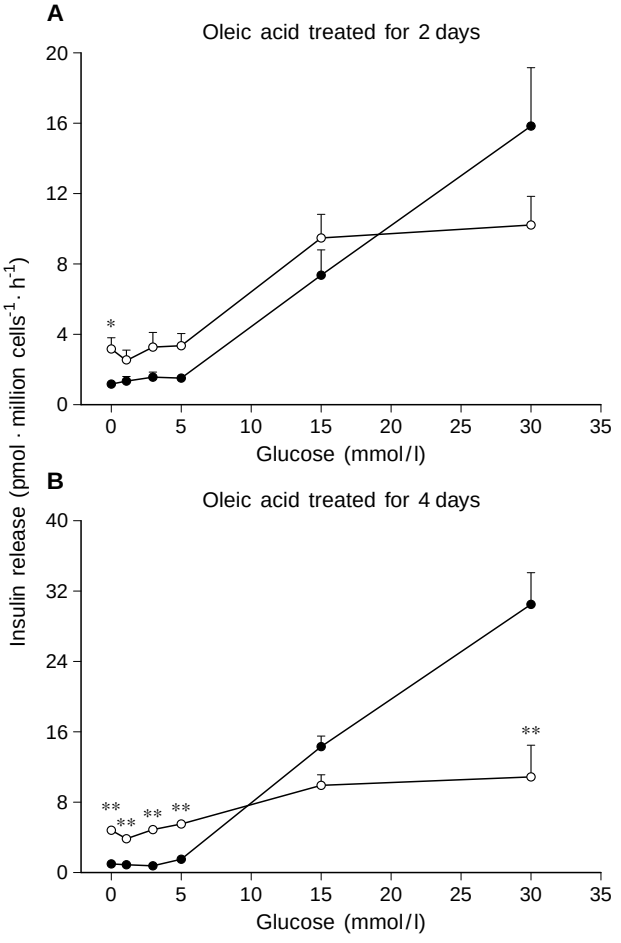

Fig. 1A, B. Effects of chronic oleic acid treatment on glucose-induced insulin release from βHC9 cells. βHC9 cells were cultured in DMEM with 2% BSA in the presence (–○–) or absence (-●-) of 2 mmol/l oleic acid for 2 days (**A**) or for 4 days (**B**). Insulin release was determined by static incubations. * $p < 0.05$, ** $p < 0.01$, compared with the controls ($n = 6$)

Effect of chronic oleic acid treatment on glucose metabolism in βHC cells. The activities of glucokinase and hexokinase in βHC9 cells were measured both in the total cell homogenate and in the cytosolic fraction. The glucokinase activity in the cell homogenate expressed in terms of U/g protein was similar to that in the cytosol. However, the specific activity of hexokinase in the cell homogenate was approximately twofold higher than that in the cytosol. In cells treated with oleic acid at different levels and for different time periods, total glucokinase activity and its glucose K_m were not different from those in the control groups (Table 1). However, the hexokinase activity was significantly increased when βHC9 cells were treated with 2 mmol/l oleic acid for 4 days (Table 2). This increase was observed both in the cell homogenate and in the cytosolic portion. The glucose K_m of hexokinase was not changed by oleic acid. This effect of oleic acid on hexokinase is dose-dependent, since treating βHC9 cells with 1 mmol/l oleic acid for

Table 1. The effect of oleic acid and glucose on glucokinase activity

Treatment	V_{max} (U/g protein)		K_m (mmol/l)
	Total homogenate	Cytoplasm	
2 days			
25 mmol/l G + 2% BSA	4.61 ± 0.24	4.76 ± 0.30	13.3 ± 1.00
25 mmol/l G + 2% BSA + 1 mmol/l OA	4.93 ± 0.35	5.08 ± 0.26	16.8 ± 1.04
25 mmol/l G + 2% BSA + 2 mmol/l OA	4.31 ± 0.31	5.22 ± 0.26	13.5 ± 1.16
4 days			
25 mmol/l G + 2% BSA	4.99 ± 0.82	4.17 ± 0.65	8.33 ± 1.53
25 mmol/l G + 2% BSA + 1 mmol/l OA	4.02 ± 0.48	4.34 ± 0.47	9.78 ± 1.91
25 mmol/l G + 2% BSA + 2 mmol/l OA	4.66 ± 0.28	4.54 ± 0.74	11.7 ± 1.83
4 days			
5 mmol/l G + 2% BSA	2.50 ± 0.20[a]	2.23 ± 0.36[a]	12.6 ± 1.27
5 mmol/l G + 2% BSA + 1 mmol/l OA	2.57 ± 0.26[a]	2.12 ± 0.43[a]	12.8 ± 1.83
5 mmol/l G + 2% BSA + 2 mmol/l OA	2.57 ± 0.20[a]	2.29 ± 0.40[a]	15.3 ± 3.49

The means ± SEM of five experiments are shown.
G, Glucose; BSA, bovine serum albumin; OA, oleic acid.
[a] $p < 0.05$, compared with that in βHC9 cells cultured in 25 mmol/l glucose with 2% BSA for 4 days

Table 2. The effect of oleic acid and glucose on hexokinase activity

Treatment	V_{max} (U/g protein)		K_m (mmol/l)
	Total homogenate	Cytoplasm	
2 days			
25 mmol/l G + 2% BSA	2.01 ± 0.10	1.30 ± 0.09	0.09 ± 0.01
25 mmol/l G + 2% BSA + 1 mmol/l OA	2.15 ± 0.08	1.39 ± 0.05	0.09 ± 0.01
25 mmol/l G + 2% BSA + 2 mmol/l OA	2.83 ± 0.08[b]	1.84 ± 0.12[b]	0.13 ± 0.02
4 days			
25 mmol/l G + 2% BSA	1.80 ± 0.30	1.22 ± 0.19	0.06 ± 0.01
25 mmol/l G + 2% BSA + 1 mmol/l OA	2.35 ± 0.34	1.39 ± 0.26	0.07 ± 0.01
25 mmol/l G + 2% BSA + 2 mmol/l OA	2.99 ± 0.51[a]	2.04 ± 0.33[a]	0.11 ± 0.03
4 days			
5 mmol/l G + 2% BSA	1.96 ± 0.17	1.02 ± 0.25	0.09 ± 0.01
5 mmol/l G + 2% BSA + 1 mmol/l OA	2.57 ± 0.17[c]	1.40 ± 0.30	0.10 ± 0.02
5 mmol/l G + 2% BSA + 2 mmol/l OA	3.79 ± 0.32[d]	2.17 ± 0.44[c]	0.14 ± 0.05

The means ± SEM of five experiments are shown.
[a] $p < 0.05$; [b] $p < 0.01$, compared with that in βHC9 cells cultured in 25 mmol/l glucose with 2% BSA for the same period of time;
[c] $p < 0.05$, [d] $p < 0.01$, compared with that in βHC9 cells cultured in 5 mmol/l glucose with 2% BSA

4 days produced only a small 30% increase of hexokinase activity (not significant statistically). The effect of chronic oleic acid treatment on hexokinase seems to be independent of the ambient glucose concentration.

Glucose usage in βHC9 cells was measured with 5-[^{3}H]-glucose. The glucose dose-dependency curve of glucose usage and the result of the kinetic analysis using the Eadie-Hofstee plot are shown in Figure 2 and Table 3. In βHC9 cells treated with 2 mmol/l oleic acid, glucose usage at 4–8 mmol/l glucose was increased approximately 20% compared with that in the controls. When the glucose concentrations were greater than 8 mmol/l, no differences in the rates of glucose usage were observed between the treatment and control groups. The Eadie-Hofstee plots indicated that there are two components of glucose usage. The high K_m component is dominant and occupies more than 90% of the glucose usage. The V_{max} of this high K_m component in βHC9 cells treated with oleic acid was the same as that of the controls; however, oleic acid treatment significantly increased the V_{max} of the low K_m component (Table 3). It is worth emphasizing that in the physiological range of glucose levels oleic acid treatment had little impact on the glucose usage.

It has been suggested that low lactate dehydrogenase activity is a characteristic feature of normal pancreatic beta cells [17]. In several insulin-secreting cell lines, the abnormal left-shift of the insulin release response to glucose stimulation is often accompanied by a marked increase in the activity of this enzyme. To test whether lactate dehydrogenase is a target for oleic acid and whether this is the cause of beta-cell dysfunction, we measured lactate production in situ. In oleic acid treated βHC9 cells, the lactate production at different glucose concentrations was indistinguishable from that in controls (Fig. 3), indicating that oleic acid has little effect on the reduction of pyruvate to lactate.

The effect of chronic oleic acid treatment on glucose oxidation in βHC9 cells was studied by measuring oxidation of ^{14}C(U)-glucose to $^{14}CO_2$ at glucose concentrations ranging from 0.06 to 32 mmol/l (Fig. 4, Table 3). The Eadie-Hofstee plot revealed the presence of two components with high and low K_m values, respectively. The high K_m component is the dominant one. In βHC9 cells exposed to 2 mmol/l oleic acid for 4 days, the V_{max} of glucose oxidation for the high K_m component was significantly reduced by more than 35% compared to the controls. At low glucose concentrations glucose oxidation rates were the same in the two groups (Table 3). Again, oleic acid treatment had no effect on the glucose K_m values of both components.

Based on the results of glucose usage, glucose oxidation and lactate production, we calculated the rate for ATP production specifically derived from glucose [8]. Two assumptions were made for this calculation: (1) all the NADH generated by glycolysis

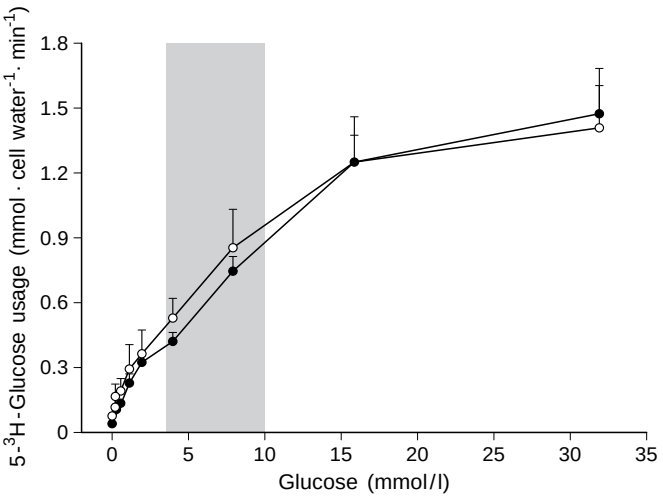

Fig. 2. Effect of chronic oleic acid treatment on glucose usage in βHC9 cells. Glucose usage was determined by a radiometric method. Concentration dependency of glucose usage in the control (-●-) and oleic acid treated (–○–) βHC9 cells is shown. The shaded area indicates the glucose concentrations in the physiological range. The Eadie-Hofstee plot was used to calculate the V_{max} and K_m values for glucose usage in these cells (see Table 3) ($n = 6$)

is channeled into mitochondrial oxidation and lactate production; and (2) the P/O ratio for mitochondrial oxidation phosphorylation is assumed to be 2.5 in both control and experimental cells and it is postulated that a long-term exposure of beta cells to fatty acids does not change the P/O ratio. The specific glucose derived ATP showed a hyperbolic glucose dependency curve in both groups of cells (Fig. 5A). At glucose concentrations lower than 15 mmol/l, there was little or no difference between the control and oleic acid-treated βHC9 cells. At 32 mmol/l glucose, glucose derived ATP generation in the oleic acid treated cells was reduced by 20% compared to that in controls. When glucose-induced insulin release is plotted as a function of specific glucose derived ATP the data for the experimental group differ greatly from that of the controls. In βHC9 cells treated with oleic acid, a low apparent rate of ATP generation was accompanied by an insulin release which was 3–6-fold higher than that in the control cells. However, at apparent high rates of ATP generation, glucose-induced insulin release was much lower than that in the controls (Fig. 5B). This plot indicates that the specific glucose derived ATP production was not the dominant controlling factor for insulin release. (Note that this interpretation rests on the assumption that fatty acid treatment does not change the P/O ratio.)

In the absence of glucose, pancreatic beta-cells utilize NEFA as the major energy source. Oxidation of endogenous fuel is apparently turned off when islets are exposed to high glucose associated with increased islet glucose metabolism. Malonyl-CoA is thought to play a crucial role in this glucose-induced fuel switch via inhibition of fatty acid oxidation [18]. This switch from fatty acids to glucose as preferred fuel may be crucial for glucose-induced insulin release [18, 19]. Chronic treatment of βHC9 cells with oleic acid may interfere with the physiological rise of intracellular malonyl-CoA due to high glucose level and may reduce the efficiency of the switch. To test this possibility, we compared oleic acid oxidation under basal condition (0 mmol/l glucose) to that at different glucose concentrations in order to obtain a measure of any inhibitory effect of glucose on fatty acid oxidation (Fig. 6). In control cells, at a glucose concentration of approximately 5 mmol/l, [1–^{14}C]-oleic acid oxidation was inhibited by 50% of the basal level. When these cells were exposed to 32 mmol/l glucose, oleic acid oxidation was further reduced to 20% of that at the basal level. In βHC9 cells chronically treated with oleic acid, the result was quantitatively different. Even though the ID_{50} for glucose was the same, oleic acid oxidation at 16–32 mmol/l glucose remained as high as 35% of that at the basal level.

Table 3. The kinetics of glucose usage, oxidation and cell respiration in βHC9 cells treated with oleic acid

Treatment	V_{max} (mmol · l cell water^{-1} · min^{-1})		K_m (mmol/l)	
	High K_m Component	Low K_m Component	High K_m Component	Low K_m Component
Glucose usage				
Controls	1.97 ± 0.25	0.15 ± 0.03	14.28 ± 0.95	0.13 ± 0.04
Oleic acid treated	1.79 ± 0.39	0.33 ± 0.09[a]	8.92 ± 2.11	0.22 ± 0.04
Glucose oxidation				
Controls	0.62 ± 0.06	0.05 ± 0.00	15.80 ± 1.74	0.15 ± 0.03
Oleic acid treated	0.39 ± 0.05[a]	0.05 ± 0.01	11.46 ± 3.35	0.21 ± 0.07
Glucose induced respiration				
Controls	0.96 ± 0.11	NT	14.48 ± 2.25	NT
Oleic acid treated	0.74 ± 0.04	NT	2.56 ± 0.18[b]	NT

The means ± SEM of six experiments are shown.
[a] $p < 0.05$, compared with that in βHC9 cells cultured in 25 mmol/l glucose with 2% BSA for the same duration (48 h);
[b] $p < 0.01$, compared with that in βHC9 cells cultured in 25 mmol/l glucose with 2% BSA for the same duration (48 h); NT, Not tested

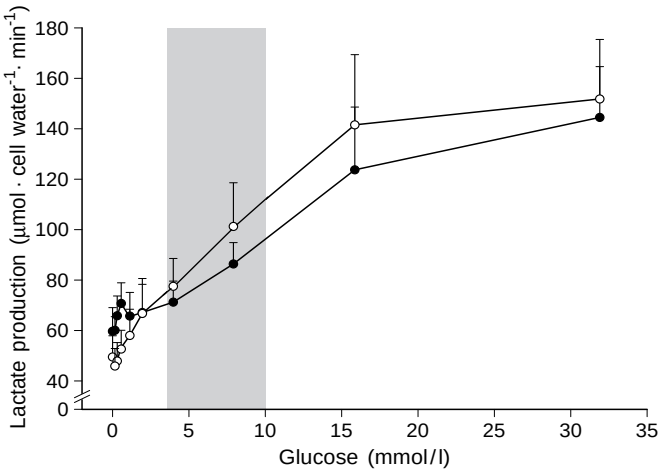

Fig. 3. Lactate production by βHC9 cells. The lactate production in controls (-●-) and oleic acid treated (–○–) βHC9 cells at different glucose concentrations was determined using a fluorometric method. The shaded area indicates the glucose concentrations in the physiological range. No statistically significant differences were found between these two groups of βHC9 cells ($n = 6$)

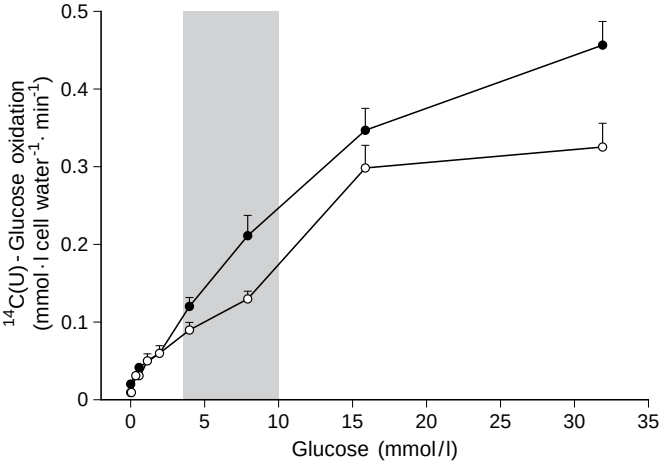

Fig. 4. Glucose oxidation in βHC9 cells. Glucose oxidation was determined by a radiometric method. The panel shows the concentration dependency of glucose oxidation in the controls (-●-) and oleic acid treated (–○–) βHC9 cells. The shaded area indicates the glucose concentrations in the physiological range. The Eadie-Hofstee plot was used to calculate the V_{max} and K_m values for glucose oxidation in these cells (see Table 3) ($n = 6$)

This was significantly higher than that in controls, indicating that chronic exposure to oleic acid interfered with the inhibitory switch of high glucose curbing fatty acid oxidation.

Effect of chronic oleic acid treatment on energy metabolism in βHC9 cells and its relationship to insulin release. To analyce the relationship between total energy generation and glucose-induced insulin release, we determined cellular oxygen consumption, which provides a measure of the sum of glucose and endogenous fuel oxidation. In oleic acid treated βHC9 cells, the total cell respiration was clearly higher compared with that in controls both in the absence of glucose and at all glucose concentrations (Fig. 7A, Table 3). The glucose K_m for stimulation of respiration was drastically lowered by NEFA treatment but the V_{max} was comparable. When insulin release was plotted as a function of total cell oxygen consumption, a very striking dissociation was found between the energy production and insulin release. This result suggests strongly that factors other than impaired energy generation must be involved in the mechanism of NEFA blockade of glucose-induced insulin release. (Note again that this interpretation rests on the assumption that the P/O ratio is not altered by the treatment with NEFA.)

Discussion

NIDDM associated with obesity is a syndrome usually explained by insulin resistance and pancreatic islet dysfunction. High level of NEFA, a characteristic of this syndrome, may play a pathogenetic role in the development of beta-cell dysfunction similar to the role of NEFA in the development of insulin resistance. It has been reported that the activity of hexokinase was increased and that pyruvate dehydrogenase activity was inhibited when pancreatic islets were treated chronically with NEFA [6, 7]. The activity changes of these two particular enzymes have been used to explain the detrimental effects of NEFA on insulin release. To further test this working hypothesis, we examined whether chronic NEFA exposure alters glucose metabolism and energy production of βHC9 cells in tissue culture. For this purpose we analysed the following key steps in glucose metabolism of these cells: (a) glucose phosphorylation, i.e. glucose sensing, (b) glycolytic flux, i.e. ATP production by substrate phosphorylation and via mitochondrial NADH shuttles, (c) glucose oxidation, i.e. a major source of the ATP derived from glucose metabolism, (d) glucose inhibition of NEFA oxidation, i.e. a measure of the efficiency of the malonyl-CoA switch from endogenous substrate to glucose as fuel, and (e) total respiration as a function of glucose, i.e. a global measure of the energy of the beta cells.

An important issue we faced was the assessment of hexokinase activity in pancreatic beta cells. There are at least two glucose phosphorylation enzymes in isolated pancreatic islet tissue. In beta cells, glucokinase is the major enzyme, whereas in non-beta cells of the pancreatic islets, hexokinase is the dominant if not the only glucose phosphorylation enzyme. Hexokinase activity in freshly isolated islet tissue is 2–4-fold higher than that of glucokinase even though non-beta cells contribute only approximately 30 % of the

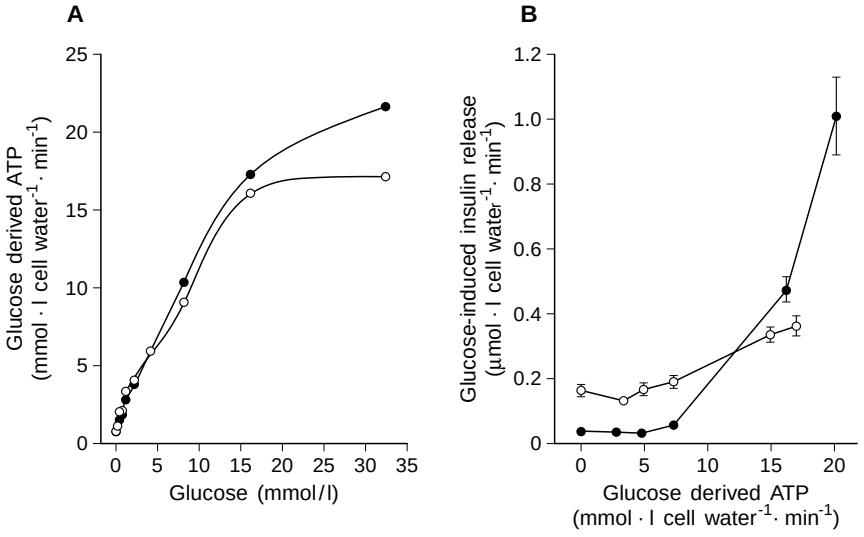

Fig. 5(A, B). The relationship between glucose-induced ATP production and glucose-induced insulin release in βHC9 cells. **A**. The glucose-induced ATP production rates in the control (-●-) and oleic acid treated (–○–) βHC9 cells were calculated based on the rate (mean values) of glucose usage, lactate production and glucose oxidation. **B.** Glucose-induced insulin release in the control (-●-) and oleic acid treated (–○–) βHC9 cells was plotted as a function of the ATP production in the same group of cells

islet cell mass [20]. Therefore, even small alterations in the number of non-beta cells or contamination with exocrine pancreas may greatly influence the outcome of hexokinase activity measurement. Heterogeneity of islet tissue thus compromises the interpretation of hexokinase measurements of intact islet tissue, especially in diabetic animals which always show morphological changes difficult if not impossible to quantify. Besides, whether chronic NEFA treatment changes the cell composition in cultured islets remains unsettled. To avoid these intractable uncertainties, samples of pure beta cells need to be used. We therefore chose the βHC9 cell line as a model to investigate this issue.

Our study of glucose metabolism in βHC9 cells chronically treated with oleic acid showed an increase in the hexokinase activity, a small left shift of the dose-dependency curve of glucose usage and a slight but significant reduction of glucose oxidation at high glucose levels. These results are in general agreement with the reports of other laboratories [6, 7]. However, when we calculated the specific glucose-derived ATP production rate, we found it to be the same in controls and experimental cells, at least at low glucose level, even though the hexokinase activity in oleic acid-treated cells had increased about 1.6-fold compared with that in controls. At glucose concentrations greater than 15 mmol/l, the ATP generation rate in the oleic acid treated cells was somewhat reduced. However, this limited reduction in the ATP production at high glucose is not correlated with the overall alteration in glucose-induced insulin release as demonstrated by a plot of insulin release as a function of glucose-derived ATP generation (Fig. 5B). We have previously compared the glucose-derived ATP generation in βHC9 and βTC3 cells. Even though these two cell lines showed different kinetics and magnitudes of insulin release as a function of the glucose level, a plot of insulin release as a function of the ATP generation rate showed that both cell lines fell on one continuous sigmoidal curve [8]. The shape of this energy production-insulin release diagram indicates that the difference of insulin release between these two cell lines is probably a result of inherent differences in energy metabolism characteristics for these cells. However, when βHC9 cells were chronically treated with oleic acid, this coherent sigmoidal relationship between energy production and insulin release was lost (Fig. 5B and 7B). Therefore, we suggest that changes in glucose dependent energy metabolism are not responsible for the high insulin release at basal glucose level and are also not the cause of the reduction of insulin release at high glucose levels. The technique of saturation transfer using phosphorus-nuclear magnetic resonance to measure the rate of ATP production [21] rather than the actual ATP levels in pancreatic beta cells will provide a critical test for the present working hypothesis.

We also studied cell respiration to evaluate the rate of total energy generation produced by oxidation of glucose and endogenous fuels including NEFA combined. Total oxygen consumption in oleic acid treated cells was 15 to 20% higher than that in the controls at all glucose concentrations studied, indicating a relatively high rate of energy generation in these cells. Considering the reduction of glucose oxidation in oleic acid treated cells, this enhancement of cellular oxygen consumption could be the result of the oxidation of enlarged lipid stores and an increased fatty

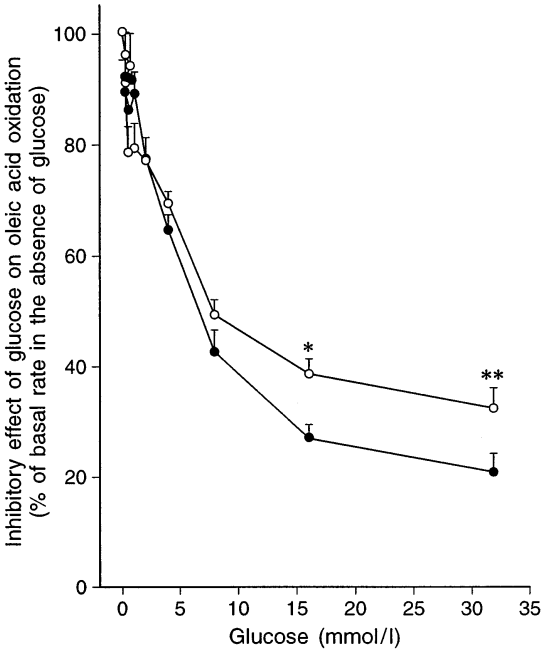

Fig. 6. Glucose inhibition of oleic acid oxidation in βHC9 cells. Oleic acid oxidation in controls (-●-) and oleic acid treated (–○–) βHC9 cells was measured using [1–^{14}C] oleic acid. The assay was started by adding 4 μmol/l of [1–^{14}C] oleic acid (0.2 μCi/ml) to fat free incubation buffer. The $^{14}CO_2$ generated under basal conditions (0 mmol/l glucose) was 11838 ± 345 dpm/10^6 cells in the control group and 6147 ± 324 dpm/10^6 cells in the oleic acid treated group. The oleic acid oxidation rates at different glucose concentrations ranging from 0.06 to 32 mmol/l are expressed as the percentage value compared with the rate at the basal condition in the absence of glucose. * $p < 0.05$, ** $p < 0.01$ compared with the value in the controls ($n = 6$)

acid pool of these cells resulting from the exposure to high oleic acid level in the culture medium. This presumption is supported by observations in experiments designed to measure oleic acid oxidation (Fig. 6). The specific radioactivity of [1–^{14}C]-oleic acid in the incubation buffer of oleic acid treated cells was reduced to approximately 50 % of that in the medium used for controls, indicating a considerable dilution with the endogenous fatty acids (data not shown). We related the cellular respiration rates to insulin release in both groups of cells and found that the presumed energy production as a sum of glucose oxidation and endogenous fuel oxidation might be dissociated from insulin release when the two experimental groups are compared (Fig. 7B). This apparent dissociation indicates that the NEFA effect on islet beta cell function must be sought in factors and processes not related to impaired energetics.

NEFA probably have a dual effect on pancreatic beta cells. Besides serving as a source of ATP, NEFA and their metabolic products may also play a direct role as signalling molecules [22, 23]. For example, long chain acyl-CoA may enhance the activity of protein kinase C [1], which may participate in glucose-induced insulin release [24]. Malonyl-CoA may have an important indirect influence on NEFA regulation of protein kinase C activity in the pancreatic islets [1]. When islets are exposed to high glucose, the content of malonyl-CoA increases, and the rate of fatty acid oxidation is reduced [25, 26]. A consequence of this reduced fatty acid oxidation in islet tissue seems to be an increased de novo synthesis of diacylglycerol and other complex lipids in beta cells which might also activate protein kinase C. In βHC9 cells chronically treated with oleic acid, we found that at high glucose levels, fatty acid oxidation was inhibited by only 65 % of the basal level while in the controls, this inhibition reached 80 % of the basal levels. The weakened inhibition of fatty acid oxidation may be an expression of an impaired rise of malonyl-CoA content possibly explained by the fact that NEFA inhibit catalytic activity and expression of acetyl-CoA carboxylase [27], the enzyme that catalyses the formation of malonyl-CoA. Whether such an oleic acid effect on malonyl-CoA and the synthesis of diacylglycerol might alter protein kinase C activity in beta cells, and thus insulin release due to glucose, remains a critical issue to be explored. Another possibility is that long-term exposure of βHC9 cells to oleic acid may increase the intracellular long-chain acyl-CoA levels to an extent that causes depletion of protein kinase C with consequences similar to those observed in phorbol-ester treated mouse islets [28].

In pancreatic islets, cyclic AMP activated signalling pathways interact with signal-transduction and modulate insulin synthesis and secretion [29, 30]. It has been reported that NEFA are able to activate adenylate cyclase and increase the formation of cyclic AMP in brain tissue [31]. Whether this effect occurs in pancreatic beta cells remains to be explored. Long-term exposure of beta cells to NEFA may result in a change of the membrane potential due to altered potassium channel activity resulting from protein kinase A modification. Hyperpolarization or depolarization may interfere with the generation of the electrical signals due to metabolic coupling factors derived from glucose. The increased hexokinase activity in oleic acid treated βHC9 cells may be a manifestation of increased cyclic AMP levels, since cAMP has been shown to increase the hexokinase I mRNA levels in cultured rat thyroid FRTL5 cells [32].

A recent study observed that long chain acyl-CoA may activate the ATP-sensitive K^+ channel and prevent channel closure [33], which is a key link in the mechanism of glucose-induced insulin release. Whether this phenomenon also occurs in pancreatic beta cells exposed to NEFA for a long period should be explored. Other factors governing insulin secretion, such as insulin biosynthesis, the process of insulin granule exocytosis, the control of increased intracellular calcium concentration and protein phosphorylation may play some role in the lipotoxicity

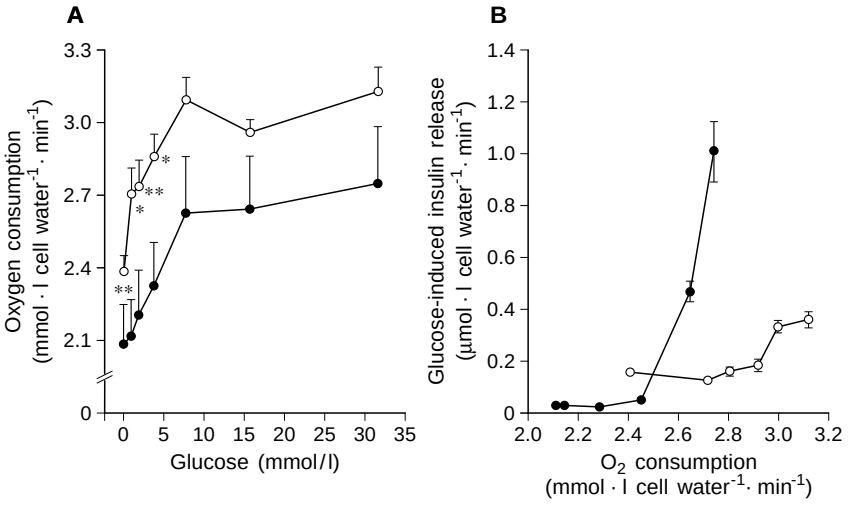

Fig. 7(A, B). Cellular oxygen consumption and its correlation with insulin release at different glucose concentrations. **A** shows the oxygen consumption in controls (-●-)and oleic acid treated(–○–) βHC9 cells at different glucose concentrations. **B.** Glucose-induced insulin release in controls (-●-) and oleic acid treated (–○–) βHC9 cells plotted as a function of the total cell oxygen consumption in the same group of cells ($n = 6$)

affecting pancreatic islets. The present investigation of glucose stimulated insulin release, does not allow conclusions to be made whether long-term oleic acid treatment influences these separate processes of insulin biosynthesis, processing, storage and regulated secretion. The long-term exposure of beta cells to fatty acids may also change the cell membrane in a subtle manner and interfere with the exocytosis of insulin. Impaired insulin output due to glucose could be caused by interference at any one of these steps. The exact site at which fatty acids act needs to be pinpointed.

Extrapolations from these model studies using tumourous beta cells in tissue culture to the situation in obese diabetic human subjects must be made with extreme caution, as highlighted by a recent study by G. Boden et al. [34]. These authors failed to demonstrate an inhibitory effect of elevated NEFA on glucose induced insulin release during a 48-h fat infusion. In fact insulin release was enhanced by 46% compared to controls with comparable hyperglycaemia. This study also points out that in vivo data in man and in the rat are comparable, which raises nagging questions about the physiological and pathophysiological relevance of the inhibitory effects of NEFA treatment in vitro.

In summary, our study suggests that impaired energy production from both glucose and fatty acid metabolism in pancreatic beta cells is not the major cause of blunted insulin release in response to glucose stimulation. Increased fatty acid oxidation may explain the high insulin release in the absence of glucose and at basal glucose level. However, other factors unrelated to energy metabolism must be identified to understand the biochemical mechanisms that cause lipotoxicity in beta cells. Among these factors, protein kinase C and cyclic AMP activated pathways are plausible candidates for further investigation.

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