EXPERIMENTAL STUDY

Intracellular depletion of insulin: a comparative study with palmitate, oleate and elaidate in INS-1 cells

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Abstract

Objective: Free fatty acids (FFAs) deplete the intracellular insulin stores of pancreatic β-cells. It has been suggested that this results from a lipotoxic dysregulation of both insulin secretion and insulin synthesis. In the present study, this hypothesis was tested within a 12-h time-course by directly relating the FFA-induced loss of intracellular insulin to corresponding parameters of insulin secretion and de novo biosynthesis. Palmitate, cis-monoenic oleate and the trans-monoenic elaidate were employed as model FFAs to elucidate potentially different effects due to chain length and configuration.

Methods: INS-1 cells were incubated for 1, 4 or 12 h with 11.2 mmol/l glucose with 200 μmol/l palmitate, oleate or elaidate and compared with non-FFA-exposed controls with respect to content and secretion of immunoreactive insulin (IRI). Biosynthesis of insulin was monitored by pulse-labeling experiments and by Northern blot analysis.

Results: IRI content dropped by 50–60% after a short-term exposure with all FFAs employed (P ≤ 0.001). It tended to recover after 12 h of treatment with oleate and elaidate but not with palmitate. FFA treatment increased insulin secretion by 25% (P ≤ 0.05) which could not account quantitatively for the intracellular loss. FFA-induced changes in insulin biosynthesis did not correlate clearly with the FFA-induced intracellular loss.

Conclusions: The FFA-induced loss of IRI is an acute effect independent of the FFA employed. It cannot be sufficiently explained by FFA-induced perturbations of IRI secretion and biosynthesis. We therefore postulate an additional FFA-triggered mechanism, e.g. intracellular IRI degradation.

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Introduction

It is generally recognized that a hypercaloric diet containing high amounts of fat (i.e. the so-called ‘western diet’) is a major cause of obesity and its related disorders such as type 2 diabetes (1). Some evidence points to long chain free fatty acids (FFAs) as being a major player in the pathophysiology of these diet-induced disorders of glucose metabolism since (i) FFA levels are typically elevated in obese subjects under a western diet and (ii) FFAs contribute not only to insulin resistance but also induce an impaired β-cell function (for reviews see 2 and 3).

Thus, a prolonged exposure of pancreatic β-cells to FFA increases the intracellular triacylglycerol content (4) and impairs (i) insulin secretion (5–9), (ii) preproinsulin biosynthesis (8, 10–12) as well as (iii) posttranslational proinsulin processing (11, 14). A fourth effect of FFA, namely the decrease of the intracellular content of immunoreactive insulin (IRI), is thought to be a consequence of the insulinotropic effects of FFA and/or an inhibited (prepro)insulin production and has been proposed to be a pathomechanism for β-cell exhaustion in type 2 diabetes (10, 15, 16).

The physiologically predominant fatty acids are the saturated hexadecanoic acid, i.e. palmitic acid, and the mono-unsaturated cis-configured Δ-9-octadecenoic acid, i.e. oleic acid (17). However, modern industrial processing of typical ‘western diet’ food has also led to an increased intake of trans-monoenic fatty acids, predominantly in the form of various positional isomers of the octadecenoic acid molecule (18, 19). In islet cell research, parallel examination of different FFAs and/or geometric isomers on the pancreatic β-cell has focused on their insulinotropic potency revealing conflicting results (9, 20–24).

Different effects of FFAs on intracellular IRI depletion and (prepro)insulin biosynthesis during short-term and prolonged exposure have not yet been elucidated. In this paper, we therefore present serial data examining
(i) secretion of IRI, (ii) the cellular content of IRI as well as (iii) preproinsulin biosynthesis and preproinsulin mRNA. As a cell model we have chosen INS-1 cells, a rat insulinoma-derived β-cell line (25), treated with (i) palmitate (hexadecanoic acid 16:0), (ii) oleate (octadecenoic acid 18:1 c9), and (iii) elaidate (octadecenoic acid 18:1 c9) during a time-course of 12 h.

Materials and methods

General study design
INS-1 cells (25) were incubated either with different FFA s (palmitate, oleate or elaidate) or in a FFA-free control medium for a time-period of 1, 4 or 12 h. Afterwards, secretion of IRI was assayed from the cellular supernatants. Final intracellular IRI content as well as the final preproinsulin biosynthesis rate were measured in the corresponding cell lysates. Cellular preproinsulin mRNA content was analyzed in separate INS-1 samples under the same conditions of exposure. A detailed description of the experimental procedures is given below.

Materials

L-[35S]-methionine (43.5 TBq/mmol) was from NEN (Boston, MA, USA). IRI levels were measured by radioimmunoassay (RIA) using the rat insulin kit from Linco (St Louis, MO, USA). A polyclonal anti-bovine insulin antiserum from Sigma-Aldrich (Taufkirchen, Germany) was used for the immunoprecipitations of newly synthesized IRI. Pansorbin was purchased from Calbiochem (La Jolla, CA, USA) and palmitic oleic and elaidic acid from Sigma-Aldrich (Taufkirchen, Germany). Fatty acid ultra-free bovine serum albumin (BSA; fraction V) and protease inhibitor cocktail (complete) were from Roche Diagnostics (Mannheim, Germany). Cellulose membrane filter (MF-Millipore 0.45 µm) and a sample manifold apparatus were from Pall Gelman (Taufkirchen, Germany). Nylon membranes (0.45 µm pore size) were purchased from Pall Gelman Laboratory (Dreieich, Germany). All other chemicals were from Merck Eurolab (Darmstadt, Germany) or from Sigma-Aldrich and were of the highest purity available.

Cell culture

The polyclonal rat insulinoma cell line INS-1 (passage nos 70–90) was used for the experiments (25). The cells were held in culture in RPMI-1640 medium 11.2 mmol/l containing glucose, 2 mmol/l L-glutamine, 1 mmol/l sodium pyruvate, 50 µmol/l β-mercaptoethanol, 100 U/ml penicillin, 100 µg/ml streptomycin and 10% (v/v) heat-inactivated fetal calf serum and incubated at 37°C/5% CO2. At a confluence of about 80%, cells were subcultured or employed for the experiments.

At the beginning of each experiment, cells were pre-incubated in six-well plates for 24 h in 500 µl methionine-deprived RPMI-1640. This pre-incubation was followed by a 1-, 4- or 12-h exposure to palmitate (hexadecanoic acid, 16:0), oleate (octadecenoic acid, 18:1 c9) or elaidate (octadecenoic acid, 18:1 c9). The FFA s were used at a total concentration of 200 µmol/l pre-complexed to 0.2% (w/v) fatty acid ultra-free BSA (n = 6.7) (26). Controls were incubated without complexed FFA in the presence of 0.2% (w/v) BSA. Thirty minutes prior to the end of incubation, 0.2 nmol L-[35S]-methionine (1.85 MBq) was added to each sample.

Analysis of IRI secretion, content and biosynthesis

At the end of the exposure time, supernatants were collected, centrifuged and kept frozen pending analysis of IRI secretion by RIA. The remaining INS-1 cells were lysed in 300 µl detergent containing buffer (50 mmol/l HEPES (pH 8.0), 0.1% (v/v) Triton X-100 plus protease inhibitor cocktail). After sonication and centrifugation at 10 000 g for 2 min to remove debris, aliquots were taken for assessment of (i) the protein content by the bicinchoninic method and (ii) the final IRI content by RIA as well as for the measurement of (iii) the total protein biosynthesis rate and (iv) biosynthesis of rat preproinsulin 2. To determine the total protein biosynthesis aliquots were treated with 10% (w/v) trichloroacetic acid (TCA) to precipitate the cellular protein. The protein precipitates were then trapped onto MF-Millipore membranes and extensively washed in 5% (w/v) TCA by flow through on a sample manifold apparatus prior to analysis for incorporated [35S] by a standard scintillation counting procedure (27). To determine preproinsulin biosynthesis, the aliquots were first subjected to immunoprecipitation against IRI as described previously (11). After additional TCA precipitation, IRI was spotted onto MF-Millipore membranes and analyzed for the amount of incorporated [35S].

Northern blot mRNA analysis

INS-1 cells were cultured in 15 cm Petri dishes to about 80% confluence and exposed for 1, 4 or 12 h to 0.2% (w/v) fatty acid ultra-free BSA with or without 200 µmol/l FFA (see above). From the cellular RNA preparations of each sample, a total of 10 µg per lane was separated by 1% agarose/formaldehyde electrophoresis and transferred to a nylon membrane by capillary elution. After UV crosslinking, the blot was probed against IRI and β-actin using the respective random-labeled cDNA probes (28). The preproinsulin mRNA content was quantified as a ratio of the densitometric intensity between the preproinsulin and the corresponding β-actin signal.
**Presentation of results**

Data are presented as means±S.E. of at least five independent experiments. Statistically significant differences were analyzed using a two-tailed Student’s t-test where \( P \leq 0.05 \) was considered significant.

**Results**

**Differences in FFA-induced IRI depletion**

The effects of palmitate, oleate and elaidate on the absolute amount of intracellular IRI were compared during a time-course of 12 h \((n = 5; \text{Fig. 1})\). In untreated control cells, the intracellular IRI content approximately doubled from the start to the end (t\(_{12\text{h}}\)) of the experiment. In contrast, additional treatment with any fatty acid (i.e. palmitate, oleate or elaidate) led to a spontaneous decline in intracellular IRI reaching the lowest observed value after 4 h of exposure (t\(_{4\text{h}}\)). At this time, the IRI content was approximately halved for all fatty acids in comparison with the control \((P \leq 0.001)\). Palmitate and especially oleate seemed to have more rapid effects when compared with elaidate. Short-term exposure of 1 h caused a tendency to a decrease for all three fatty acids which was found to be statistically significant for the oleate-exposed cells \((P \leq 0.05)\). Between t\(_{4\text{h}}\) and t\(_{12\text{h}}\) the intracellular IRI content appeared to recover in those INS-1 cells which were treated either with oleate or elaidate, so there was no longer a significant difference from the untreated controls after 12 h of exposure. In contrast, palmitate-exposed INS-1 cells showed – if at all – the slowest recovery: their intracellular IRI content remained at about one-third of that of the control cells even after 12 h of exposure \((P \leq 0.05)\).

**FFA-induced IRI secretion does not account for the intracellular IRI loss**

When considering IRI secretion in the identical INS-1 cells, we found a higher insulin secretion after 1 h of oleate exposure with 0.5±0.2 ng IRI/mg lysate protein compared with 0.3±0.1 ng IRI/mg lysate protein in the control. Overall (i.e. for all FFAs employed at all time-points) there was a moderate insulinotropic effect with a mean surplus of 0.07±0.03 ng IRI/mg lysate protein corresponding to an average relative increase of 25% above control \((P \leq 0.05)\). We could not detect any significant insulinotropic differences between the three different FFAs employed.

In absolute terms, the moderate FFA-induced surplus in insulin secretion could not account for the FFA-induced loss in intracellular insulin (Fig. 2). Thus, the amount of the additionally secreted IRI never exceeded 10% (mean ±1.1%) of the corresponding intracellular IRI loss at any time-point and independently of the FFA employed.

**FFA-induced effects on preproinsulin biosynthesis and intracellular IRI depletion**

The incorporation of \([^{35}\text{S}]\) into immunoprecipitable insulin (IRI) during a 30-min labeling period with L-\([^{35}\text{S}]\)-methionine served as a measure for the biosynthetic rate of preproinsulin at the different time-points stated above. The amount of \([^{35}\text{S}]\) incorporated into TCA precipitates reflected the rate of general protein biosynthesis (Table 1). Surprisingly, after 1 h of incubation (t\(_{1\text{h}}\)), elaidate induced a significant rise in the biosynthetic rates of both IRI (44±12%) and general protein (18±4%). There was a tendency for oleate to cause an increase in IRI biosynthesis, whereas the palmitate-induced changes in IRI and protein biosynthesis were negligible within ±7% above control (Table 1). After 4 h of exposure, palmitate and oleate caused a tendency to an inhibited IRI and total protein biosynthesis synthesis which was not the case for elaidate (Table 1). Finally, after 12 h of exposure to palmitate, there was a significant decrease in both IRI and protein synthesis by 20±7% and 11±3% compared with untreated controls. Oleate and elaidate did not cause any significant effects. In comparing different time-points of incubation with each other we found a significant decline in IRI synthesis for palmitate exposure between t\(_{1\text{h}}\) and t\(_{12\text{h}}\) (Table 1).

**Specific effects of FFA on preproinsulin biosynthesis**

By correcting the \([^{35}\text{S}]\) incorporation into IRI relative to \([^{35}\text{S}]\) incorporation into total lysate protein, it is possible...
to indicate the specific effects (i.e. above those on general protein biosynthesis) on insulin biosynthesis (27). Therefore, we normalized IRI biosynthesis to de novo protein synthesis and termed this specific IRI biosynthesis. To elucidate whether fatty acid treatment simultaneously changed insulin gene transcription and/or cellular mRNA content, we also measured preproinsulin mRNA content standardized to β-actin mRNA under the conditions described above (n = 7). After 1 h of exposure (t 1h) we observed a trend-wise increase in specific IRI synthesis in oleate-treated cells of 42 ± 22%: Palmitate and elaidate treatment did not lead to changes in specific IRI biosynthesis; preproinsulin mRNA content remained at the same level as control for all fatty acids (Table 2). After 4 h of incubation, preproinsulin mRNA content significantly decreased in elaidate-exposed cells by 27 ± 11%, whereas no concomitant change could be noted for palmitate and oleate (Table 2). After 12 h of incubation, no clear

Table 1 FFA-induced effects on the biosynthetic rate of preproinsulin and total protein. Preproinsulin and total protein biosynthesis were measured as [35S]-methionine incorporation after a 30-min pulse into IRI immunoprecipitates and TCA precipitates respectively, in the presence of palmitate, oleate and elaidate for 1, 4 and 12 h. The results, corrected for equal amounts of cellular protein, are given as the relative change compared with the corresponding untreated control. The data represent means ± S.E. of five independent experiments.

<table>
<thead>
<tr>
<th>Change from control (%)</th>
<th>Palmitate</th>
<th>Oleate</th>
<th>Elaidate</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 h IRI biosynthesis</td>
<td>7 ± 3</td>
<td>42 ± 13</td>
<td>44 ± 13*</td>
</tr>
<tr>
<td>Total protein biosynthesis</td>
<td>0 ± 5</td>
<td>14 ± 4</td>
<td>18 ± 4*</td>
</tr>
<tr>
<td>4 h IRI biosynthesis</td>
<td>−20 ± 11</td>
<td>−9 ± 5</td>
<td>10 ± 19</td>
</tr>
<tr>
<td>Total protein biosynthesis</td>
<td>14 ± 8</td>
<td>−15 ± 6</td>
<td>6 ± 6</td>
</tr>
<tr>
<td>12 h IRI biosynthesis</td>
<td>−20 ± 7*</td>
<td>0 ± 16</td>
<td>−11 ± 9</td>
</tr>
<tr>
<td>Total protein biosynthesis</td>
<td>−11 ± 3*</td>
<td>−7 ± 5</td>
<td>−4 ± 5</td>
</tr>
</tbody>
</table>

* P < 0.05 compared with concurrent control.

Table 2 FFA-induced effects on specific preproinsulin biosynthesis and preproinsulin mRNA content. Specific preproinsulin biosynthesis was defined as the ratio between IRI biosynthesis and total protein biosynthesis in INS-1 cells (compare Table 1). Cellular preproinsulin mRNA was assayed in cell lysates by densitometric Northern blot analysis and corrected for cellular β-actin mRNA. The results are given as absolute change in % compared with untreated controls. The values are expressed as means ± S.E. of five and seven independent experiments.

<table>
<thead>
<tr>
<th>Change from control (%)</th>
<th>Palmitate</th>
<th>Oleate</th>
<th>Elaidate</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 h Specific insulin biosynthesis</td>
<td>5 ± 9</td>
<td>43 ± 16</td>
<td>20 ± 12</td>
</tr>
<tr>
<td>Preproinsulin mRNA</td>
<td>0 ± 7</td>
<td>−12 ± 9</td>
<td>−10 ± 9</td>
</tr>
<tr>
<td>4 h Specific insulin biosynthesis</td>
<td>−14 ± 7</td>
<td>7 ± 6</td>
<td>11 ± 16</td>
</tr>
<tr>
<td>Preproinsulin mRNA</td>
<td>−3 ± 6</td>
<td>−19 ± 16</td>
<td>−27 ± 11*</td>
</tr>
<tr>
<td>12 h Specific insulin biosynthesis</td>
<td>−15 ± 9</td>
<td>−3 ± 13</td>
<td>14 ± 27</td>
</tr>
<tr>
<td>Preproinsulin mRNA</td>
<td>−14 ± 10</td>
<td>−10 ± 7</td>
<td>−9 ± 16</td>
</tr>
</tbody>
</table>

* P < 0.05 compared with concurrent control.

Figure 2 Qualitative relation between insulin secretion and insulin content. Cumulative IRI secretion was assayed by measuring IRI in the supernatants of INS-1 cells treated as described with palmitate, oleate and elaidate. FFA-induced changes in IRI secretion (solid bars) and IRI content (open bars) are given as absolute difference ± S.E. from the concurrent untreated controls after 1, 4 and 12 h of incubation. The values represent means ± S.E. of five independent experiments. *P ≤ 0.001 when compared with concomitant controls.
effects, either on specific IRI synthesis or on the preproinsulin mRNA content, could be observed (Table 2).

Discussion

FFAs were initially described as being a secretagogue to acutely potentiate glucose-stimulated insulin release in the pancreatic β-cell (29). It has therefore been assumed that the FFA-induced intracellular insulin depletion is an immediate consequence of these insulino-tropic effects (10, 12).

However, in the present study, the FFA-induced surplus IRI secretion (i.e. the amount above the secretory release of untreated controls) is negligible compared with the amount of FFA-induced intracellular loss. Therefore, the additional insulino-tropic potency of FFAs can be ruled out as the primary cause of FFA-induced intracellular insulin depletion (Fig. 2). Here it must be noted that potential differences in the insulino-tropic potency of oleate and palmitate (not found to be significant in the present study, but previously reported to be significant (20 –23)) would also be without impact on the intracellular IRI content, just because of their absolute magnitude.

Palmitate and oleate are reported to diminish preproinsulin biosynthesis in rat pancreatic islets; this may occur either as a part of an unspecific inhibition of the overall protein biosynthesis or as a specific effect on proinsulin biosynthesis (10 –12, 30).

We recently reported (16) a reduction not only in the total but also in the newly synthesized intracellular IRI in oleate-exposed INS-1 cells. Since there was also a shift towards mature insulin within the IRI pool we suggested an FFA-induced lack of incompletely processed IRI and concluded that, in the presence of oleate, β-cellular preproinsulin biosynthesis cannot adequately compensate for the extra- and intracellular IRI consumption (16).

It must be noted that the degree of post-translational processing as a surrogate parameter for proinsulin biosynthesis (16) points to methodological difficulties in the attempt to definitively separate de novo proinsulin biosynthesis from other possible intracellular proceedings, e.g. intracellular insulin degradation (31 –33). Similarly, a comparison between the intracellular IRI content (measurable in absolute terms) and the biosynthetic rate of IRI (only measurable in relative terms) has to be correlative and therefore does not allow a statement about the compatibility of the two parameters in regard to their absolute extent.

In the present study, we did not find a concordant inhibition of the biosynthetic rate between 30 and 60 min of FFA exposure which could conclusively explain the acute IRI depletion after 1 h of FFA exposure. Moreover, in the very short term, the monoenic oleate and elaidate caused an increase rather than a decrease of proinsulin biosynthesis. The elaidate-induced increase was accompanied by an increased biosynthesis of total protein (Table 1), whereas the stimulatory effect of oleate appeared to be IRI specific (Table 2). Such acute IRI-specific effects are reminiscent of a synergistic translational effect of oleate and the peroxisome proliferator-activated receptor γ-agonistic troglitazone which we recently observed in an in vitro translational setting with preproinsulin mRNA from INS-1 cells (34). From the present biosynthetic data it must be concluded that short-term FFA-induced changes in proinsulin biosynthesis cannot be the reason for intracellular IRI depletion.

During prolonged FFA exposure (i.e. 12 h) only palmitate (not oleate and elaidate) caused an inhibition of proinsulin biosynthesis as part of an inhibited total protein biosynthesis. This could explain the different time-course of palmitate versus oleate/elaidate in regard to the intracellular insulin content (Fig. 1).

Neither the saturated palmitate nor the monoenic oleate influenced the cellular preproinsulin mRNA content after up to 12 h of exposure, which is in accordance with previous findings with oleate under hyperglycemic conditions (35) and palmitate, which suppressed preproinsulin mRNA only after long-term (i.e. 48 h) exposure (8). It must be noted that there was also no significant change in proinsulin biosynthesis despite a significant decline of the cellular preproinsulin mRNA content after 4 h of elaidate exposure. These findings suggest that the quantitative supply of preproinsulin mRNA is not the primary limiting factor for preproinsulin biosynthesis translation.

Taken together, the results in the present study characterized the FFA-induced depletion of intracellular IRI as an early and pronounced effect that exceeds the insulino-tropic potency of FFAs by far. Unspecific, e.g. detrimental detergent effects or pro-apoptotic effects (36) of the FFAs themselves are unlikely (i) because there was no large increase in supernatant IRI after FFA exposure compared with untreated controls and (ii) there was a stable to again increasing insulin content after prolonged (i.e. > 4 h) exposure to all FFAs (Fig. 1).

The FFA-induced perturbances in insulin secretion and in insulin biosynthesis are not sufficient to explain the rapid loss of intracellular insulin that we observed. A third IRI-depleting effect caused by FFA has therefore to be postulated. Possible candidates could be (i) the phenomenon of crinophagy/degradation (31 –33), (ii) compartmentalization effects or (iii) FFA-mediated protein modifications which could influence the functionality or detectability of IRI molecules in the immunologic assay procedures employed.

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