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Synergistic effects of troglitazone and oleate on the translatability of preproinsulin mRNA from INS-1 cells

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Abstract

Glitazones are known to modulate fatty acid-induced effects on insulin secretion in the pancreatic β-cell. The present study focused on combined effects of troglitazone and oleate on preproinsulin (PPI) biosynthesis. Insulin-producing INS-1 cells were incubated for 4 hr at 11.2 mM glucose in the presence (O^+) or absence (O^-) of 200 μM oleate with (T^+) or without (T^-) 10 μM troglitazone. After cell lysis, cytoplasmic RNA was extracted and employed for Northern blotting and corresponding *in vitro* translation. Compared with untreated controls (CTRL = O^-/T^-), the cellular content of PPI-mRNA from cells which had been simultaneously treated by troglitazone and oleate (O^+/T^+) was significantly diminished ($O^+/T^+ = 75 \pm 10\% \times$ CTRL; $P = 0.015$). The PPI-mRNA content from those cells which had been exclusively exposed either to oleate (O^+/T^-) or troglitazone (O^-/T^+) did not significantly differ from that of the untreated controls. In spite of that decreased PPI-mRNA content, *in vitro* translation revealed the highest yield of newly synthesized PPI in RNA samples from those cells which had been simultaneously exposed to oleate and troglitazone before ($O^+/T^+ = 1.6 \pm 0.3 \times$ CTRL; $P = 0.01$). It is concluded that troglitazone and oleate synergistically affect the translational rate at the level of the PPI-mRNA molecule.

Keywords: Pancreatic β-cells; INS-1 cells; Insulin biosynthesis; *In vitro* translation; Free fatty acids; Thiazolidinediones; Glitazones; Troglitazone

1. Introduction

Thiazolidinediones are going into widespread clinical use for the treatment of obesity-related type 2 diabetes [1–4]. As ligands of the nuclear peroxisome proliferator-activated receptor γ (PPARγ) thiazolidinediones affect the cellular gene expression in a pleiotropic manner that leads to an improved peripheral tissue sensitivity to insulin as demonstrated by numerous clinical and molecular studies [5–9]. Unlike the peripheral insulin sensitive tissue, the effects of thiazolidinediones on the insulin-producing pancreatic β-cell have been less characterized although PPARγ is also highly expressed in islets of Langerhans [10,11].

Prolonged exposure of pancreatic islets to free fatty acids (FFAs) increases the intracellular triacylglycerol content and thereby impairs insulin secretion as well as the fine-tuned balance between β-cell mitogenesis and apoptosis [12–16]. Thus, in obese homozygous (fa/fa) Zucker rats it has been demonstrated that the chronic hyperlipacidemic status leads to an excess accumulation of triacylglycerol in the pancreatic islets with consequence of an impaired insulin secretion [13]. The thiazolidinedione compound troglitazone is able to reverse the triacylglycerol accumulation in these fat-laden islets of (fa/fa) Zucker rats [17]. By lowering the triacylglycerol deposits it thereby restores the impaired insulin secretion and also prevents from morphological abnormalities such as apoptosis [17,18]. Finally, as troglitazone exerts no effects in islets of corresponding lean wild type (+/+) Zucker rats without triacylglycerol overload, it has been suggested that thiazolidinediones counteract the chronic effects of FFAs and is directly effective against lipotoxicity [17]. By using the pancreatic β-cell line INS-1, another very recent study has found similar ameliorative effects of troglitazone in terms of FFA-impaired, glucose-stimulated

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Abbreviations: FFAs, free fatty acids; IRI, immunoreactive insulin (i.e. proinsulin, conversion intermediates and insulin); PPAR, peroxisome proliferator activator receptor; PPI, preproinsulin; TCA, trichloroacetic acid.

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insulin secretion [19], whereas the very same study as well as a study using purified pancreatic β-cells [20] contrarily describe a troglitazone-induced aggravation in terms of FFA-induced necrosis/apoptosis by troglitazone.

The present study focuses on insulin biosynthesis as another important parameter for proper β-cell function. Prolonged elevated levels of FFA are known to have lasting effects not only on insulin secretion and cell turnover (see earlier description) but also on insulin biosynthesis [12,14,21–23]. The study designed to test possible ameliorative effects of troglitazone on oleate deteriorated insulin production provides evidence for a translational regulation of PPI biosynthesis which might be different from that of glucose [24–26].

2. Materials and methods

2.1. Reagents

L-[^{35}S]Methionine (43,5 TBq mmol^{-1}) was from NEN. A polyclonal anti-bovine insulin antiserum from Sigma–Aldrich was used for all immunoprecipitations of IRI [14]. Pansorbin^{TM} was purchased from Callbiochem-Novabiochem. Fatty acid ultra-free BSA (Fraction V) and protease inhibitor cocktail (Complete^{TM}) were from Roche Diagnostics. Cellulose membrane filter (MF-Millipore^{TM} 0.45 μm HA) and a sample manifold apparatus were from Millipore. Nylon membranes (0.45 μm pore size) were purchased from Pall Gelman Laboratory. For *in vitro* translation studies [26], a commercially available system (Retic Lysate IVT^{TM}) from Ambion was used. Troglitazone (±5-(4-(6-hydroxy-2,5,7,8-tetra-methylchroman-2-ylmethoxy)benzyl)-2,4-thiazolidinedione) was kindly provided by Sankyo. All other chemicals were from Sigma–Aldrich or from Merck Eurolab and of the highest purity available.

2.2. Cell culture

INS-1, a glucose sensitive pancreatic β-cell line [24] of passage #60 to #80 was used in the experiments. The INS-1 cells were maintained at 11.2 mM glucose in a modified RPMI 1640 medium containing 2 mM L-glutamine, 1 mM sodium pyruvate, 50 μM β-mercaptoethanol, 100 U mL^{-1} penicillin, 100 μg mL^{-1} streptomycin, and 10% (v/v) heat-inactivated fetal calf serum and incubated at 37° in 5% CO_2 as described [24]. At a confluence of about 80%, cells were subcultured or employed for the experiments.

2.3. Concentration of oleate and troglitazone

Oleate was used in a standard concentration of 200 μM with a concomitant BSA (fatty acid ultra-free BSA, fraction V) concentration of 0.2% (w/v). Solubilization of oleate was mediated by ethanol ($c_{final[Et-OH]} = 0.4\%$ (v/v)) which was also added to the non-oleate-containing samples [12–14]. Troglitazone was used in a standard concentration of 10 μM with DMSO as vehicle of solubilization ($c_{final[DMSO]} = 0.01\%$ (v/v)). The latter was added in the same concentration to the non-troglitazone-containing samples [17].

2.4. Analysis of proinsulin and total protein biosynthesis in intact cells

INS-1 cells were subcultured in 6-well plates to about 80% confluence. The cells were then preincubated for 20 hr in 500 μL serum-free and methionine-deprived RPMI 1640 medium supplemented with 0.2% (w/v) fatty acid-free BSA, 1 nM trijodthyronine and 4 μgL^{-1} IGF-1. The preincubation was followed by a 4-hr labeling period with 1.85 MBq of L-[^{35}S]methionine in the presence or absence of oleate and/or troglitazone. At the end, supernatants (500 μL) were collected, centrifuged and kept frozen pending analysis by RIA and immunoprecipitation, respectively. The INS-1 cells were washed twice in ice-cold PBS and then lysed in 300 μL detergent-containing buffer (50 mM HEPES (pH 8.0), 0.1% (v/v) Triton X-100 plus protease inhibitor cocktail). After additional sonication and a centrifugation at 10,000 *g* for 2 min to remove debris, aliquots were taken for assessment of (i) the total intracellular protein content by the bicinchoninic method and of (ii) the total protein synthesis. For the latter, aliquots were treated by 10% (w/v) trichloroacetic acid (TCA) to precipitate the cellular protein. The protein precipitates were trapped onto MF-Millipore^{TM}-membranes and extensively washed in 5% (w/v) TCA by flow through on a sample manifold apparatus prior to analysis for incorporated [^{35}S] by a standard scintillation counting procedure [27]. The remainder of the radiolabeled cell lysate (250 μL) was subjected to a specific immunoprecipitation against immunoreactive insulin (IRI) as described earlier [28,29]. After additional chemical precipitation by TCA (see earlier description), IRI was spotted onto MF-Millipore^{TM}-membranes and analyzed for the amount of incorporated [^{35}S]. To assess the secretion of newly synthesized IRI, the same immunoprecipitation technique was applied in the secretory supernatants of the INS-1 cells.

2.5. Northern blot mRNA analysis

INS-1 cells were cultured in ∅15 cm petri dishes to about 80% confluence. After a 20-hr period in a serum-free, methionine-containing RPMI 1640 medium, cells were incubated for 4 hr in the presence or absence of oleate with or without troglitazone. Subsequently, cells were lysed and cytoplasmic RNA was prepared according to the method of Gough [30]. A total of 10 μg RNA per lane was separated by 1% agarose/formaldehyde electrophoresis and transferred to a nylon membrane by capillary elution. After UV crosslinking, the blot was analyzed for

PPI-mRNA and actin mRNA using the respective random-labeled cDNA probes [31].

2.6. Analysis of proinsulin and total protein biosynthesis by in vitro translation

For *in vitro* translation a commercially available system containing nuclease-treated reticulocyte lysate from rabbits (Retic Lysate IVT™) was employed [32]. A total of 11 μg RNA per 25 μL-assay was incubated for 1 hr at 37° in the presence of 0.75 MBq L-[^{35}S]methionine. Afterwards, the amount of incorporated [^{35}S] was measured in (i) TCA-precipitated total protein and in (ii) separately immunoprecipitated PPI. Each individual RNA was tested in triplicate.

2.7. Analysis of the expression of PPARα, γ and δ by reverse transcription PCR

From INS-1 lysates, RNA was prepared [30] and first-strand DNA generated. For PCR the PPAR-specific primers were employed as described by Zhou *et al*. [10]: *PPARα:* 5′-AAGCCATCTTCACGATGCTG-3′ (sense, 1321–1340) and 5′-TCAGAGGTCCCTGAACAGTG-3′ (antisense, 1811–1830). *PPARγ*: 5′-TCCGTGATGGAAGACCACTC-3′ (sense, 190–209) and 5′-CCCTTGCATCCTTCACAAGC-3′ (antisense, 502–521). *PPARβ/δ*: 5′-CTTCAGTGACATCATTGAGC-3′ (sense, 1221–1240) and 5′-GACAGCATGAACAGGAAGTG-3′ (antisense, 1751–1760). The first-strand DNA samples were amplified for 35 cycles under following parameters: 94° for 45 s, 52° for 30 s, and 72° for 45 s. Presence and size of the obtained PCR products were analyzed on a ethidium bromide-stained 2% agarose gel.

2.8. Statistical analysis

Data are presented as means ± SE of at least six independent experiments. Differences were analyzed by a non-parametric Mann–Whitney *U*-test. Multiplicity adjustment was performed according to the Bonferroni procedure considering a *P*-value less than 0.017 statistically significant [33].

3. Results

3.1. Expression of PPARα, γ and δ in INS-1 cells

By employing specific primers against the reverse transcribed DNA of mRNA from the PPAR isoforms α, γ and β/δ, reaction products were identified in the predicted sizes of 509 bp (PPARα), 331 bp (PPARγ), and 548 bp (PPARβ/δ). This is in accordance to previous findings both in rodent and in human pancreatic islets [10,11] and confirms a very recent INS-1 study of Kawai *et al*. [19] in terms of PPARγ expression (Fig. 1).

3.2. Secretory output of IRI

INS-1 cells were cultured for 4 hr in the presence or absence of oleate with or without troglitazone. Overall, untreated INS-1 cells (CTRL) released 75 ± 20 ng IRI mg^{-1} lysate protein. Those INS-1 cells which had been exclusively exposed to oleate (O^+/T^-) released during the same time 320 ± 130 ng IRI mg^{-1} lysate protein which was in average 2.5-fold more than in the untreated

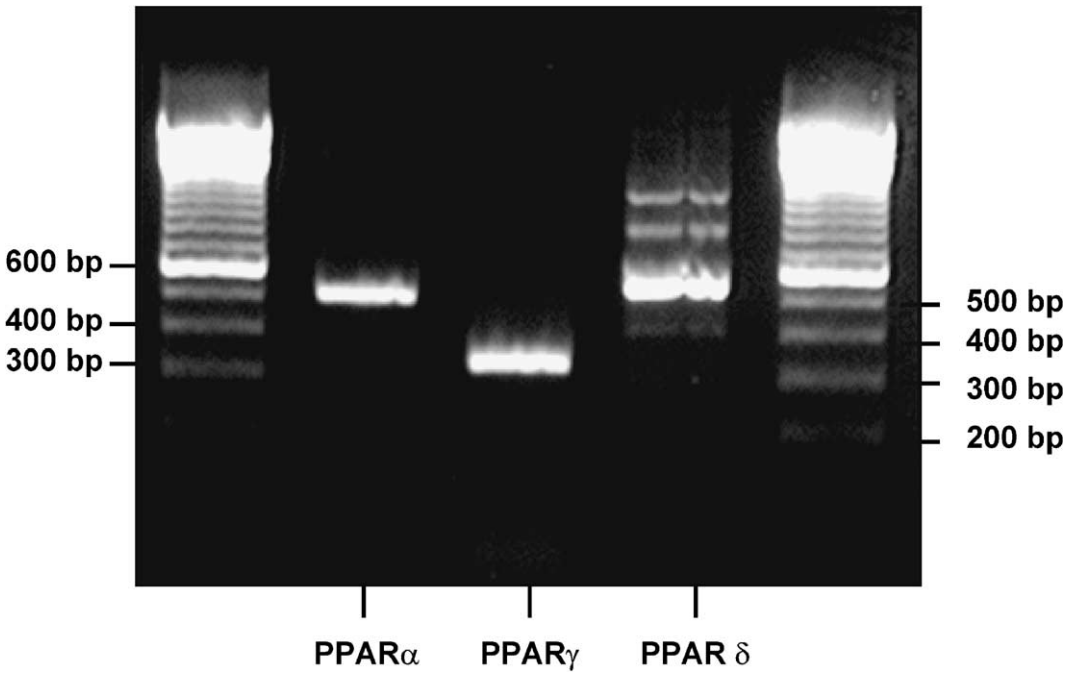

Fig. 1. Expression of the mRNA of the prevalent PPARα, PPARγ, and PPARβ/δ in INS-1 cells. After reverse transcription and PCR of the first-strand DNA with specific primers [10], reaction products were identified in the predicted sizes of 509 bp for PPARα, 331 bp for PPARγ, and 548 bp for PPARβ/δ.

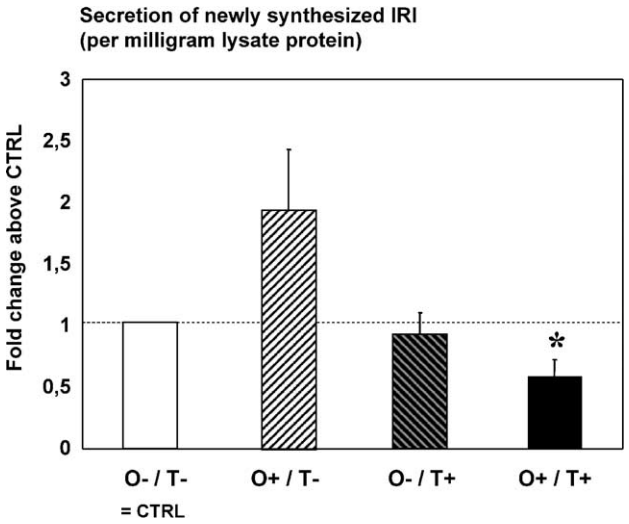

Fig. 2. Secretion of newly synthesized IRI in the presence or absence of oleate and troglitazone. INS-1 cells were radiolabeled (50 μCi L-[^{35}S]methionine) for 4 hr at 11.2 mM glucose in the presence (O^+) or absence (O^-) of 200 μM oleate with (T^+) or without (T^-) 10 μM troglitazone. From the cellular supernatants a specific immunoprecipitation against IRI was performed and incorporation of [^{35}S]methionine into IRI was assessed by β-emission scintillation counting. Data are corrected for equal amounts of cellular lysate protein and expressed as a fold change relative to the respective control cells. Values are means ± SE of eight independent experiments. Statistical significance against CTRL (∗) is adjusted according to Bonferroni with $P_{\text{significant}} \leq 0.017$.

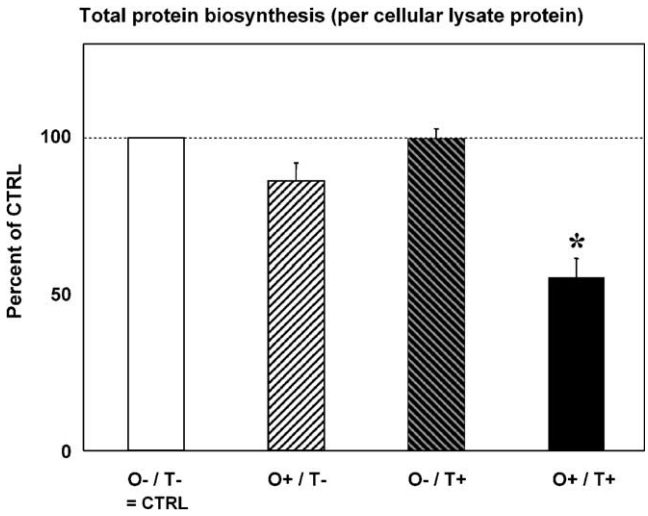

Fig. 3. Total protein biosynthesis of INS-1 cells in the presence or absence of oleate and troglitazone. INS-1 cells were incubated and radiolabeled as described for Fig. 2. After cell lysis, incorporation of [^{35}S]methionine into total lysate protein was quantified by β-emission scintillation counting of TCA precipitates. Data are corrected for equal amounts of cellular lysate protein and expressed as a percentage decrease relative to the respective control cells. Values are means ± SE of nine independent experiments. Statistical significance against CTRL (∗) is adjusted according to Bonferroni with $P \leq 0.017$.

controls. Cells which had been incubated in the sole presence of troglitazone (O^-/T^+) secreted 280 ± 220 ng IRI mg^{-1} lysate protein, cells which had been simultaneously exposed to both oleate and troglitazone (O^+/T^+) 180 ± 70 ng IRI mg^{-1} lysate protein.

The secretory insulin output was also monitored by scintillation counting of [^{35}S] into newly synthesized IRI from the cellular supernatants (Fig. 2). The secretory release of the newly synthesized IRI generally tallied with the aforementioned total IRI release. Thus, INS-1 cells which had been solely exposed to oleate (O^+/T^-) secreted almost double the amount of newly synthesized IRI than did the corresponding controls ($P = 0.01$). No difference could be observed between those cells which had been solely treated by troglitazone (O^-/T^+) and their respective untreated controls. Under simultaneous incubation with oleate and troglitazone (O^+/T^+), the secretion of newly synthesized IRI was inhibited by 40 ± 15% in comparison to the untreated controls ($P = 0.01$).

3.3. Newly synthesized total protein and IRI in intact INS-1 cells

The amount of [^{35}S] incorporation per milligram of total lysate protein served as a measure for total protein *de novo* biosynthesis (Fig. 3). Compared with the untreated controls (CTRL), in cells which had been exclusively incubated with oleate (O^+/T^-), the [^{35}S] incorporation into total protein was diminished by 15 ± 5% ($P = 0.05$). Also, no significant difference could be observed between those INS-1 cells which had been exclusively exposed to troglitazone (O^-/T^+) and their respective untreated controls. However, cells which had been simultaneously incubated with oleate and troglitazone (O^+/T^+) showed a reduction of [^{35}S] incorporation into total cellular protein by 45 ± 5% when compared to their untreated controls ($P = 0.001$).

The [^{35}S] incorporation into the IRI of the cellular lysates corrected for total cellular lysate protein served as a measure for the biosynthesis of IRI (rat IRI 2) [34]. In accordance with the findings of the [^{35}S] incorporation into total protein (see earlier description) both the cells which had been solely exposed to oleate (O^+/T^-) and those which had been simultaneously treated by oleate and troglitazone (O^+/T^+) showed a blunted [^{35}S] incorporation into the intracellular IRI in comparison to their untreated controls. Thereby, incorporation was diminished by 20 ± 10% in O^+/T^- ($P = 0.05$) and by 30 ± 10% in O^+/T^+ ($P = 0.004$). No significant difference was observed between the cells which were solely treated by troglitazone (O^-/T^+) and their untreated controls.

By correcting the [^{35}S] incorporation into IRI relative to the [^{35}S] incorporation into the total lysate protein, it is possible to bring out specific effects on (prepro)insulin biosynthesis which are above those on general protein biosynthesis [14,27] (Fig. 4). Both oleate *per se* (O^+/T^-) and troglitazone *per se* (O^-/T^+) failed to provide such specific effects on IRI production, whereas the combined exposure of oleate and troglitazone (O^+/T^+) elicited a specific stimulation of IRI production in comparison

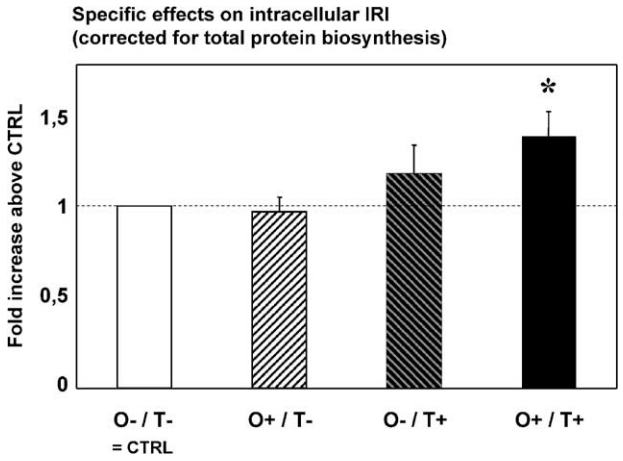

Fig. 4. Newly synthesized IRI in INS-1 cells after oleate and/or troglitazone treatment—specific effects on PPI biosynthesis above that of general protein biosynthesis. Radiolabeling with L-[^{35}S]methionine was performed as described for Fig. 2. IRI was precipitated from cellular lysates and analyzed for incorporated [^{35}S]methionine by β-emission scintillation counting. The incorporation of [^{35}S]methionine into IRI was corrected for equal amounts of [^{35}S]methionine into total lysate protein (compare Fig. 3) which brings out specific effects on PPI biosynthesis above that on general protein biosynthesis [27]. Data are expressed as a fold change relative to respective untreated control cells and represent the means ± SE of nine independent experiments. Statistical significance against CTRL (∗) is adjusted according to Bonferroni with $P \leq 0.017$.

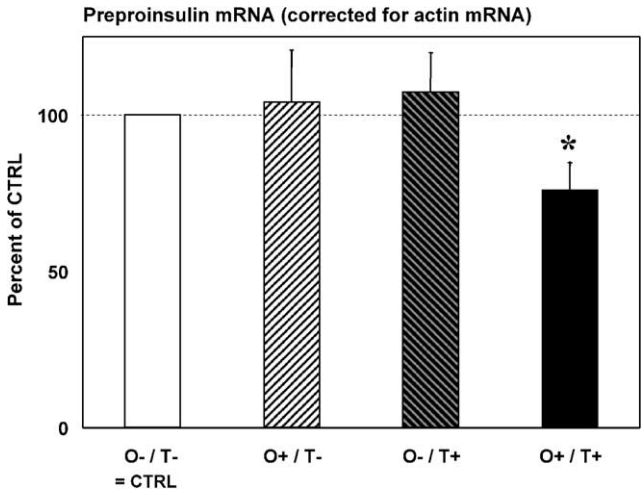

Fig. 5. Oleate- and troglitazone-induced effects on PPI-mRNA levels. INS-1 cells were incubated for 4 hr at 11.2 mM glucose in the presence (O^+) or absence (O^-) of 200 μM oleate with (T^+) or without (T^-) 10 μM troglitazone. After cell lysis and extraction of cytoplasmic RNA, Northern blot analysis was performed for PPI-mRNA and actin mRNA. The data are expressed as a percentage decrease relative to the respective control cells and represent the means ± SE of 10 independent experiments. Statistical significance against CTRL (∗) is adjusted according to Bonferroni with $P \leq 0.017$.

to the untreated control ($\text{IRI-spec}_{O^+/T^+} = 1.4 \pm 0.1 \times \text{IRI-spec}_{CTRL}$; $P = 0.008$).

3.4. Cellular content of PPI-mRNA

To further analyze specific effects on IRI production, Northern blot analysis for PPI-mRNA and actin mRNA was performed (Fig. 5). Compared with the untreated controls, INS-1 cells which had been solely exposed to oleate (O^+/T^-) and those which had been solely exposed to troglitazone (O^-/T^+) showed no significant changes in their cellular PPI-mRNA content. Only after a simultaneous exposure to oleate and troglitazone (O^+/T^+) the cellular level of PPI-mRNA was decreased by 25 ± 10% ($P = 0.015$).

3.5. In vitro translation of PPI from RNA of INS-1 lysates

A higher degree of IRI synthesis relative to general protein biosynthesis in O^+/T^+ (Fig. 3) notwithstanding the lower content of PPI-mRNA (Fig. 5) suggested possible changes in the translatability of the PPI-mRNA molecules. This hypothesis was further tested by an *in vitro* translation system [32] to compare the translatability of isolated RNA from differently treated INS-1 cells. After a 1-hr incubation of isolated INS1-RNA in nuclease-treated reticulocyte lysate with [^{35}S]methionine, the amount of [^{35}S] incorporated in newly synthesized IRI (i.e. rat PPI 1 and 2, [34]) relative to the [^{35}S] incorporation into total protein served as the measure for possible changes in PPI biosynthesis translation (Fig. 6). Thereby, the translational yield of PPI did not significantly differ between the untreated controls (CTRL) and the samples with either RNA from solely oleate treated cells (O^+/T^-) or RNA from solely

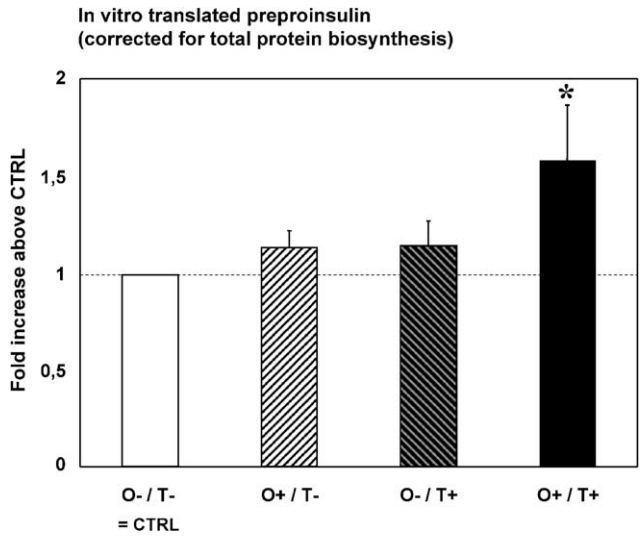

Fig. 6. Specific effects on the translatability of PPI-mRNA from INS-1 cells. INS-1 cells were treated as described for Fig. 4. Aliquots of 11 μg cytoplasmic RNA were subjected to an *in vitro* translation system in the presence of 20 μCi L-[^{35}S]methionine. Both [^{35}S]methionine incorporation into PPI and [^{35}S]methionine incorporation into total protein were quantified after TCA precipitation and immunoprecipitation, respectively. The yield of newly translated PPI corrected for equal amounts of newly translated total protein is depicted. Data are expressed as a fold change relative to the respective untreated control cells. Values are the means ± SE of seven independent experiments each performed in triplicates. Statistical significance against CTRL (∗) is adjusted according to Bonferroni with $P \leq 0.017$.

troglitazone-treated cells (O^-/T^+). Only that RNA which had been isolated from INS-1 cells after simultaneous exposure to oleate and troglitazone (O^+/T^+), led to a specific increase in PPI translation (IRI-vitro$_{O^+/T^+}$ = 1.6 ± 0.3 × IRI-vitro$_{CTRL}$; $P = 0.01$).

4. Discussion

Chronically elevated FFAs are believed to play a crucial role in the pathogenesis of certain forms of type 2 diabetes by both inhibiting insulin-stimulated peripheral glucose uptake and contributing to pancreatic dysfunction (concept of lipotoxicity) [13,35,36]. Detrimental effects of FFA on the pancreatic β-cell involve an impairment of insulin secretion and production [12,21–23,28,29,37–40] as well as other metabolic and morphological abnormalities [41,42] which might be in the long-term connected with induction of apoptosis [16,43] and/or inhibition of mitogenesis [15].

Thiazolidinedione derivatives such as troglitazone (CS-045, ±5-(4-(6-hydroxy-2,5,7,8-tetra-methylchroman-2-ylmethoxy)benzyl)-2,4-thiazolidinedione) are known to modulate cell function in a pleiotropic manner mainly through PPARs which form a subfamily of the nuclear receptor gene family [44,45]. Although thiazolidinediones (such as rosiglitazone and pioglitazone) are routinely employed in the treatment of type 2 diabetes their effects on the pancreatic β-cell are far less characterized than the molecular mechanisms leading to the improvement of peripheral insulin insensitivity. The isotype PPARγ is the predominant receptor for thiazolidinediones. It is described to be expressed both in rodent and in human pancreatic islets [10,11]. Consequently, its mRNA [19] as well as the mRNA of the other isotypes PPARα and PPARδ (=PPARβ) are also expressed in the polyclonal rat β-cell line INS-1 which has been employed for the present study (Fig. 1).

Under physiological circumstances the pancreatic β-cell maintains a stable balance between insulin secretion and production as secreted insulin is concomitantly replenished by a corresponding stimulation of PPI biosynthesis [25–27]. This fine-tuned balance is disturbed by a sustained exposure to FFAs where the intracellular insulin content of the β-cell is gradually decreasing [12,21]. That depletion of the intracellular insulin stores is thought to be a consequence of (i) an FFA-induced increase of the basal insulin secretion and (ii) an FFA-induced inhibition of the PPI biosynthesis [14,29]. The physiologically important oleic acid ($C_{18:1\ cis}$) and palmitic acid ($C_{16:0}$) are known to diminish general protein biosynthesis in rat pancreatic islets both under normoglycemic (5.6 mM) and hyperglycemic (27 mM) conditions [12,29] implying that an FFA-induced inhibition of PPI biosynthesis may occur as a part of an unspecific inhibition of the overall protein biosynthesis in the pancreatic β-cell. Nonetheless, there is also a body of evidence for a specific modulation of PPI biosynthesis by FFA either on the translational level and/or on the level of cellular PPI-mRNA content [14,22,23].

With the assessment of the incorporated [^{35}S]methionine into TCA-precipitable protein, the present study confirms an unspecific inhibition of total protein biosynthesis in the presence of oleate (Fig. 3). We found the effect even aggravated in the additional presence of troglitazone although troglitazone *per se* induced no changes in total protein biosynthesis in INS-1 cells.

Beside these overall effects on total protein biosynthesis, our present labeling experiments in intact INS-1 cells unraveled a specific intracellular up-regulation of newly synthesized IRI after the combined exposure to oleate and troglitazone (Fig. 4) which was the starting point for further investigations.

As a possible explanation for that intracellular increase we found a diminished extracellular release of newly synthesized IRI in INS-1 cells that had been treated simultaneously with oleate and troglitazone (Fig. 2). That effect which still needs further characterization has been also observed after a long-term exposure (48 hr) of INS-1 cells to oleate and troglitazone [19]. It is reminiscent of the insulin sparing effect of troglitazone reported by Shimabukuro *et al*. in fat-laden (fa/fa) Zucker rats [17] and somehow resembles the paradox reversal of the insulintropic potency of FFA under high glucose concentrations [12].

A second reason for the specific increase of newly synthesized IRI in INS-1 cells after combined exposure to oleate and troglitazone is a specific increase of IRI biosynthesis which we further examined by Northern blot analysis and additional *in vitro* assays: effects of FFA on the cellular content of PPI-mRNA are conversely discussed and might depend on the chain length and/or on the degree of saturation of the employed fatty acid [14,22,23]. As far as the *cis*-configurated, monounsaturated oleic acid is concerned, we have previously reported an increased cellular content of PPI-mRNA in rat pancreatic islets under normoglycemic conditions [14], while Ritz-Laser *et al*. found no oleate-induced effect on the PPI-mRNA content at hyperglycemic conditions [23]. By employing the polyclonal β-cell line INS-1 at hyperglycemic 11.2 mM glucose, we here found no significant change in the cellular content of PPI-mRNA after sole oleate exposure (Fig. 5) so that the findings are consistent with the findings of Ritz-Laser *et al*. [23]. Although troglitazone *per se* did not alter the cellular content of PPI-mRNA, there was a significant decrease after the combined incubation with oleate and troglitazone. Gremlich *et al*. [22] reported a decrease of PPI-mRNA after sole treatment of rat pancreatic islets with the saturated palmitic ($C_{16:0}$) acid. Thereby, the authors emphasize that the mitochondrial oxidation of the fatty acid component has to be ensured [22]. Troglitazone is known to increase mitochondrial oxidation in pancreatic β-cells [17]. It might be, therefore, possible that the intracellular fate of oleic acid is channeled by troglitazone

towards an oxidative metabolism which then results in a decreased PPI-mRNA content.

From the present data, combined incubation with oleate and troglitazone resulted in a decrease of cellular PPI-mRNA (Fig. 5) but led to a specific increase of newly synthesized insulin in INS-1 lysates (Fig. 4). These—at a first glance—contradictory findings point to possible alterations in the translatability of the PPI-mRNA which we further pursued by employing *in vitro* translation [32]. Using L-[^{35}S]methionine as a tracer, rat PPI 1 and 2 [34,46] can be both assessed due to the lack of posttranslational processing under *in vitro* conditions. RNA extracted from those INS-1 cells which had been treated simultaneously with troglitazone and oleate led to the highest translational yield of PPI (1/2) notwithstanding the decreased proportion of PPI-mRNA in the cytoplasmic RNA (Fig. 6). These results strongly suggest elements of translational control within the PPI-mRNA molecule modulated by the combined action of troglitazone and oleate. The data also arise the question of a functional connection between the translatability of the single PPI-mRNA molecule and its stability. Assuming that the decreased cellular mRNA content is a consequence of an increased decay [23], it might be worth to further focus on mRNA elements such as the polyadenylation site or the m^{7}GTP-cap (m^{7}G(5′)ppp(5′)N) which have been described to influence both translatability and stability of mRNA molecules [47,48].

Taken together, the present study characterizes a novel effect on the β-cellular PPI biosynthesis translation which is triggered in the simultaneous presence of oleate and troglitazone. Measuring different biosynthetic parameters (i.e. (i) specific PPI biosynthesis in intact INS-1 cells, (ii) cellular PPI-mRNA content, (iii) *in vitro* translation of PPI), the effect could be traced back and localized on the level of the single PPI-mRNA molecule. We here employed the polyclonal rat insulinoma cell line INS-1 in order to get enough biological material for performing quantitative and functional RNA assays. Given the lack of a glucose regulated proinsulin biosynthesis in INS-1 cells [24], the molecular mechanisms of the troglitazone/oleate-altered PPI translation might be different from those of glucose-stimulated PPI biosynthesis translation [25–27]. From the point of a clinical view a specific modulation of PPI biosynthesis translation by troglitazone in oleate exposed INS-1 cells appears to be generally ameliorative in adipogenic type 2 diabetes, however, it has to be pointed out that the global effects on insulin production—which comprise not only the specific stimulatory effect on PPI translation (Figs. 3 and 6) but also the aspect of a generally blunted protein synthesis (Fig. 2) and a decreased PPI-mRNA content (Fig. 4)—are not so easily classifiable.

The present study focused on the outcoming effects on β-cellular insulin production and was not intended to be an investigation of the initial molecular signaling steps induced by oleate and/or troglitazone. Since INS-1 cells are shown to express all the prevalent PPARα, PPARβ, and PPARγ (Fig. 1) [19] and since the genes of many important enzymes in the FFA metabolism are endowed with respective PPAR response elements [10], the here described synergistic effects of troglitazone and oleate might be primarily interpreted as a troglitazone/PPARγ-mediated channeling of oleate's pleiotropic action on insulin production. Nonetheless, alternative pathways (e.g. PPAR-independent action of troglitazone) are also generally conceivable [19]. Finally, as both FFA and troglitazone are tightly binding to albumin, the unbound FFA and/or troglitazone concentrations may be elevated in a condition when both components are added to the medium due to competitive effects. Likewise other *in vitro* studies dealing with FFA and thiazolidinediones [19,20], we did not perform the ADIFAB method [49] and/or high performance frontal analysis [50] to measure FFA and/or troglitazone so that we cannot unerringly exclude that a simultaneous incubation with FFA and/or troglitazone evolves a higher free troglitazone or FFA concentration.

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