Accepted Manuscript

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PII: S0303-7207(17)30399-4

DOI: 10.1016/j.mce.2017.07.026

Reference: MCE 10025

To appear in: *Molecular and Cellular Endocrinology*

Received Date: 10 May 2017

Revised Date: 17 July 2017

Accepted Date: 24 July 2017

Please cite this article as: Lehmphul, I., Hoefig, C.S., Köhrle, J., 3-Iodothyronamine reduces insulin secretion *in vitro* via a mitochondrial mechanism, *Molecular and Cellular Endocrinology* (2017), doi: 10.1016/j.mce.2017.07.026.

3-iodothyronamine reduces insulin secretion *in vitro* via a mitochondrial mechanism

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Abbreviated Title: Glucoregulation by 3-T_1AM *in vitro*

Key terms: thyroid hormone metabolites, 3-iodothyroacetic acid, MIN6 cells, beta(β)-cell, pancreas, diabetes mellitus type I

Word count: 5.416

Number of figures/tables and Supplemental figures/tables: 5 / 3

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Disclosure Statement: The authors have nothing to disclose.

Abstract

Purpose: 3-iodothyronamine (3-T_1AM), a decarboxylated and deiodinated thyroid hormone metabolite, leads at pharmacological doses to hypoinsulinemia, hyperglucagonemia and hyperglycemia *in vivo*. As the pancreatic Langerhans islets express thyroid hormone transmembrane transporters (THTT), we tested the hypothesis that not only plasma membrane-mediated 3-T_1AM binding to and activation of G-protein coupled receptors, but also 3-T_1AM metabolite(s) generated by 3-T_1AM uptake and metabolism might decrease glucose-stimulated insulin secretion (GSIS).

Methods: Murine pancreatic β-cells MIN6 were characterized for gene expression of THTT, deiodinases and monoamine oxidases. 3-T_1AM uptake and intracellular metabolism to the corresponding 3-iodothyroacetic acid were analysed by liquid-chromatography tandem mass spectrometry (LC-MS/MS) at different time points in cells as well as the conditioned medium. Mitochondrial activity, especially ATP-production, was monitored real-time after 3-T_1AM application using Seahorse Bioanalyzer technique. Effect of 3-T_1AM on GSIS into the culture medium was assayed by ELISA.

Results: MIN6 cells express classical THTT, proposed to transport 3-T_1AM, as well as 3-T_1AM metabolizing enzymes comparable to murine primary pancreatic islets. 3-T_1AM accumulates in MIN6 cells and is metabolized by intracellular MaoB to 3-iodothyroacetic, which in turn is rapidly exported. 3-T_1AM decreases mitochondrial ATP-production concentration dependently. GSIS is diminished by 3-T_1AM treatment. Using LC-MS/MS, no further 3-T_1AM metabolites except 3-iodothyroacetic were detectable.

Conclusions: This data provides a first link between cellular 3-T_1AM uptake and regulation of mitochondrial energy metabolism in ß-cells, resulting in reduced insulin secretion. We conclude that MIN6 is an appropriate cell model to study 3-T_1AM-dependent (intra-)cellular biochemical mechanisms affecting insulin production *in vitro*.

Introduction

Thyronamines (TAM) are a new class of signalling molecules, influencing brain and cardiac function as well as energy metabolism (Hoefig, Zucchi and Koehrle, 2016). Although combined deiodination and decarboxylation of the thyroid hormones (TH) L-Thyroxine (T4) and L-T3 would produce nine possible TAMs, only 3-T_1AM, 3,5-T_2AM and T_0AM have been detected via LC-MS/MS *in vivo* in human, rodent and pig serum and tissues (Braulke, Klingenspor, DeBarber et al., 2008,Hoefig, Köhrle, Brabant et al., 2011,Scanlan, Suchland, Hart et al., 2004,Saba, Chiellini, Frascarelli et al., 2010). Repeated 3-T_1AM application in pharmacological dosage (10 or 25 mg/kg body weight) results in reduction of body weight due to a loss of fat mass caused by its lipolytic and anti-lipogenic action (Haviland, Reiland, Butz et al., 2013). In animal models 3-T_1AM rapidly switches energy source usage from carbohydrate to fat oxidation accompanied by delayed protein degradation (Braulke et al., 2008). Metabolic turnover as well as oxygen consumption are reduced, with $F_{(0)}/F_{(1)}$-ATPase and complex III (Cumero, Fogolari, Domenis et al., 2012) reported as direct mitochondrial targets of 3-T_1AM action. 3-T_1AM influences glucoregulatory processes, resulting in hypoinsulinemia, hyperglycemia and hyperglucagonemia (Venditti, Napolitano, Di Stefano et al., 2011,Klieverik, Foppen, Ackermans et al., 2009,Regard, Kataoka, Cano et al., 2007). In addition, a clinical study showed a direct correlation of serum concentrations of 3-T_1AM with glycosylated hemoglobulin (HbA_1c; 23 patients) as well as significantly elevated 3-T_1AM serum concentrations in diabetic vs. non-diabetic patients (7 vs. 8 patients) (Galli, Marchini, Saba et al., 2012). Impact of 3-T_1AM on glucose homeostasis may therefore have physiologic and pathophysiologic relevance. 3-T_1AM reaches tissue concentrations higher than T_3 und T_4 (Saba et al., 2010,Hoefig, Wuensch, Rijntjes et al., 2015). 3-T_1AM binds to the serum protein ApoB100 but not to the serum distributor proteins of classical TH. This might provide at the same time a mechanism for selective 3-T_1AM uptake by cells expressing LDL-receptor. Cellular uptake of 3-T_1AM is not mediated via the classical THTT MCT8 and MCT10 (Scanlan et al., 2004), and some more transporter candidates are in discussion. 3-T_1AM is *in vivo and in vitro* rapidly metabolized via monoamine oxidases (Mao) at the alanine-side chain to yield its corresponding thyroacetic acid (3-TA_1, Fig. 1) (Hackenmueller and Scanlan, 2012,Wood, Geraci, Nilsen et al., 2009). This process diminishes the biological availability

of 3-T_1AM in the circulation and target tissues. Until now, it is not completely understood whether metabolites from 3-T_1AM contribute to the metabolic effects described for 3-T_1AM. So far Hoefig et al. showed lack of thermoregulatory and cardiovascular effects of 3-TA_1 upon single (50 mg/kg, i.p.) or repeated (5 mg/kg, i.p. for 7 days) injection *in vivo* (Hoefig, Jacobi, Warner et al., 2015) while a vasodilatory action of its precursor 3-T_1AM was reported in mice (Gachkar, Oelkrug, Martinez-Sanchez et al., 2017). In contrast, Musilli et al. suggested that both 3-T_1AM and 3-TA_1 play a role in the stimulation of memory acquisition and reduce the threshold to pain stimuli in mice (i.c.v., 0.4 µg/kg) by activating the histaminergic system (Musilli, De Siena, Manni et al., 2014). 3-T_1AM effects partly disappear when inhibiting the enzymatic conversion to 3-TA_1 by Mao inhibitors (Ghelardoni, Chiellini, Frascarelli et al., 2014,Manni, De Siena, Saba et al., 2012), although direct application of 3-TA_1 is not able to restore 3-T_1AM effects (Hoefig et al., 2015). One may assume that part of the effects so far described for 3-T_1AM are caused by 3-TA_1. It's still unclear if the intracellular metabolism of 3-T_1AM to 3-TA_1 is needed to exert effects, using 3-T_1AM as vehicle for across the plasma membrane. For 3-TA_1 no transport mechanism has been described so far. If 3-TA_1 contributes to 3-T_1AM effects one may separate the latter from its metabolite by inhibiting MAO, the metabolizing enzymes, to provoke 3-T_1AM dependent effects only. To distinguish effects of 3-T_1AM from 3-TA_1 several specific and unspecific Mao inhibitors are in use. Iproniazid is a non-selective aminoxidase inhibitor, whereas pargylin and chlorgylin are specific inhibitors of MaoA and MaoB (Jiang, Li, Liu et al., 2015). In addition, semicarbazide-sensitive aminoxidase (Ssao) is specifically inhibited by aminoguanidin as well as semicarbazide. Conversion of 3-T_1AM to 3-TA_1 is completely blocked by iproniazid, but not by pargylin or semicarbazide (Saba et al., 2010,Orsi, Frascarelli, Zucchi et al., 2011). Using Mao inhibitors for *in vivo* experiments it came apparent, that some formerly described effects of 3-T_1AM vanish. Hepatic glucose production in HepG2 cells after 3-T_1AM-exposition (1 µM) was not any longer measurable in presence of iproniazid. Paradoxically, direct stimulation with 3-TA_1 had no effect at all. 3-T_1AM-dependent hyperglycemia was completely absent or significantly changed when using chlorgylin (Manni et al., 2012,Manni, De Siena, Saba et al., 2012).

The aim of this project was to study the 3-T_1AM dependent regulatory mechanisms leading to decreased insulin secretion in a pancreatic β-cell line. As mitochondria are one subcellular compartment that can be modulated by 3-T_1AM, we wanted to test if such an interaction could be linked to reduced insulin secretion *in vitro*. Using intact murine β-cells as *in vitro* system, we focussed on cellular uptake and intracellular metabolism of 3-T_1AM in context of mitochondrial activity and insulin secretion.

Material and methods

All chemicals used were of the highest grade of purity and from the following suppliers: Carl Roth (Karlsruhe, Germany), Merck (Darmstadt, Germany), Roche Diagnostics (Mannheim, Germany), Serva Feinbiochemikalien (Heidelberg, Germany), Sigma-Aldrich Chemie (Steinheim, Germany), Thermo Fisher Scientific Inc. (Waltham, MA, USA) and VWR International (Hannover, Germany). The following substances were kindly provided by Thomas S. Scanlan, OHSU, Portland, USA: 3-T_1AM, $^2H_4$-3-T_1AM, ^{15}N-3,5-T_2, 3-TA_1. Further internal standards $^{13}C_6$-T_4, $^{13}C_6$-T_3, $^{13}C_6$-rT_3 and $^{13}C_6$-3,3'-T_2 were procured from Isoscience LLC (King of Prussia, PA, USA). All mentioned TH, TH metabolites (THM) as well as internal standards were dissolved in pure dimethylsulfoxide (DMSO). DMSO concentration in the described experiments was at any time below 0.1%.

Cell culture

MIN6 cells were kindly provided by Dr. Miyazaki (Institute for Medical Genetics, Kumamoto, Japan) and tested negative concerning mycoplasma. Cells were cryo-conserved in passage 14. MIN6 pancreatic β-cell culture was maintained in DMEM (4.5 g/l glucose, 4 mM L-glutamine, 10 % FBS, 0.0005 % mercaptoethanol) as described before (Miyazaki, Araki, Yamato et al., 1990). Experiments were performed until passage number 25. Before every experiment cells were grown for three passages on 100 nM sodium selenite to induce Dio activity. 24 hrs prior to the performed experiments, culture medium was changed to deplete cells from high glucose and TH (DMEM 1.0 g/l glucose, 40 mM L-glutamine, FBS-free, 0.0005 % mercaptoethanol). Cells in continuous culture were split 1:3-1:5, every 4-6 days never overgrowing 70-80 % confluence to maintain β-cell function.

RNA extraction

After the experiment cells were directly lysed in a 6 well with 0.5 ml Trizol (Peqlab, Erlangen, Germany) and stored at -20 °C until further use. After thawing and before further treatment solution was left standing at RT for 10 min. A Teflon-sphere was added to each sample and shaken for 5 min, 15/s in a tissue lyser. 0.1 ml Chloroform was added and mixed with use of the tissue lyser (15 sec, 15/s), following 2-3 min incubation step at RT. Samples were centrifuged for 10 min, 12.000 g,

RT. Further steps in RNA preparation were performed as recommended by the supplier. RNA, used for cDNA-synthesis was digested with RQ1 RNase-free DNAse (Promega, Madison, WI, USA) to deplete genomic DNA.

Transporter- and enzyme expression in MIN6 cells and pancreatic mouse islets

Primary murine islets were prepared by a protocol modified after Gotoh *et al.* (Gotoh, Maki, Kiyoizumi et al., 1985), followed by RNA isolation and cDNA synthesis. cDNA was kindly provided from the German Institute of Human Nutrition, Department of Experimental Diabetology. cDNA of 4-6 animals was pooled resulting in three individual cDNA pools with comparable concentration.

cDNA synthesis and real-time qPCR

cDNA synthesis was carried out using iScript Select cDNA Synthesis Kit (Bio-Rad, Munich, Germany) following instructions provided by the manufacturer. Real-time qPCR was performed in a Bio-Rad Laboratories iCycler using Absolute qPCR SYBR Green Mix (Abgene, Epsom, UK). Amplification was started with an initial step (15 min 95 °C und 2 min 95 °C), followed by 40 cycles (30 sec 95 °C / 45 sec 58 °C / 30 sec 72 °C) and a final extension step (3 min 72 °C). iCyclers software generates Ct-values (cycle of threshold) and data-analysis followed the $2^{-\Delta Ct}$ method for calculation (Hellemans, Mortier, De Paepe et al., 2007). Hypoxanthin-Guanin-Phosphoribosyltransferase (*HPRT*) was chosen as housekeeping gene, because it is not regulated via classical TH, as well as the Ct-values are comparable to analysed genes of interest. Specificity of primers was tested after qPCR by analysing fragment length with agarose electrophoresis. Tested primers for gene expression are listed in Supplemental Table 1.

3-T_1AM uptake assay in MIN6 cells

MIN6 cells were seeded at a density of 300,000 cells / 2 ml (6 well plate) for uptake assays and allowed to grow till day five. Medium was exchanged for overnight (ON) incubation with FBS-free DMEM. Assays were performed on day six. Medium was exchanged to HEPES-containing Krebs-Ringer-buffer (KRBH; 119 mM NaCl, 4.74 mM KCl, 1.19 mM KH_2PO_4, 25 mM $NaHCO_3$, 2.54 mM

$CaCl_2$, 1.19 mM $MgCl_2$, 10 mM HEPES; pH 7.4, (Ishihara, Asano, Tsukuda et al., 1993)) ± 200 µM iproniazid or the corresponding DMSO control for 20 min. Iproniazid was washed off and cells were incubated with 100 nM 3-T_1AM diluted in KRBH. Incubation was performed at 37 °C for 0, 2.5, 5, 10, 15, 20, 30 min. After the incubation time plate was shaken to ensure equal distribution of agents. The supernatant was collected, spun down at 4 °C (2,000 rpm, 5 min) to remove cells and 200 µl supernatant was stored for analysis via LC-MS/MS. Cellular uptake-reaction was stopped by washing each well with ice cold KRBH. Cell culture plates with MIN6 cells were immediately put on dry ice and stored at -80 °C until further analysis.

Glucose stimulated insulin secretion (GSIS)

MIN6 cells were seeded at a density of 200,000 cells / 1 ml in 12 well plates in culture media for two days. Then cells were starved ON in FBS-free culture medium. At the beginning of GSIS cells were washed with KRBH and pre-incubated in KRBH with 0.1 mM glucose ± 200 µM iproniazid or the corresponding DMSO control for 2 hrs at 37°C and 5% CO_2. Thereafter, cells were incubated for 1 h with 0.5 ml KRBH/well (2.8 mM glucose) containing 10 or 100 nM 3-T_1AM, the corresponding DMSO concentration (negative control) or 30 mM KCl (positive control). GSIS was stopped by removing the incubation medium. Plates were washed with phosphate buffered saline (PBS), shock frozen on dry ice and stored at -80°C until protein determination. Incubation media were centrifuged at RT at 2,000 rpm, 5 min to reduce detached cells. Supernatant was transferred to a clear safe-lock reaction tube and stored at -80°C until insulin determination. Insulin was measured with insulin (mouse) ultrasensitive ELISA (DRG Instruments GmbH, Germany). For insulin determination from cell culture supernatants, insulin containing KRBH was diluted 1:10 in calibrator solution supplied with the used ELISA.

Seahorse Bioanalyzer

MIN6 cells were seeded at a density of 15,000 cells / 100 µl (96 well Seahorse Bioanalyzer cell culture plate) in cell culture medium. Culture medium was removed and replaced with FBS-free culture medium for ON incubation. At day two, culture medium was removed and replaced by

198 Seahorse Assay medium (unbuffered DMEM5030, 2 mM L-glutamine, ± 2.8 mM Glucose, pH 7.0).
199 For determination of the different mitochondrial functions (see calculations in Supplemental Table 2)
200 oligomycin (1.5 µM), carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP; 1.0 µM),
201 antimycin (0.5 µM), rotenon (1.5 µM) were used at the indicated final concentrations and following
202 the standard assay manual provided by the manufacturer (Seahorse Bioscience, Billerica, MA,
203 USA).
204
205 **Extraction for LC-MS/MS Analysis**
206 **1. Cells in culture**
207 Cell culture plates were thawed on ice. Cells were scraped in 200 µl buffer (250 mM D(+)-
208 saccharose, 20 mM HEPES, 0.5 M EDTA, pH 7,4) and afterwards sonicated. Samples (180 µl) were
209 extracted following the indicated protocol (Piehl, Heberer, Balizs et al., 2008), but acetone protein
210 precipitation was performed ON at -20 °C. After extraction procedure residues were reconstituted in
211 50 µl ddH_2O:methanol:acetic acid (90:10:1) mixed, centrifuged and stored at -20 °C until LC-MS/MS
212 analysis.
213
214 **2. Cell culture supernatant**
215 Stored cell culture supernatants were thawed, vortexed and centrifuged again, in order to precipitate
216 cell debris (500 g, 10 min, 4 °C). 180 µl cell culture supernatant, equalling the cell lysis volume, was
217 extracted. TMBE (methanol (MeOH)-tert-butyl methyl ether)-extraction was performed as recently
218 published (Rathmann, Rijntjes, Lietzow et al., 2015). After extraction procedure residues were
219 reconstituted in 100 µl ddH_2O:methanol:acetic acid (90:10:1) and mixed, centrifuged and stored at -
220 20 °C until LC-MS/MS analysis.

221 **Protein normalization for LC-MS/MS**
222 LC-MS/MS and GSIS data were normalized for protein content to control for variations in cell
223 content per cell culture well. 1:10 vol/vol aliquot of cells in homogenization buffer was used to
224 determine protein content in triplicate (Bio-Rad Protein Assay Dye Reagent Concentrate, Bio-Rad

225 Laboratories, Munich, Germany) using 96 well plate format and following the suppliers instructions.
226 For quantification a BSA-standard curve was used (Bradford-standard, Bio-Rad Laboratories,
227 Munich, Germany).
228
229 **Statistics and data handling**
230 Used statistic tests, number of replicates within and between experiments are indicated in the
231 corresponding figure legends. Data analysis was performed using GraphPad Prism 4.

Results

Cell viability upon 3-T_1AM and 3-TA_1 treatment

Before starting exposure experiments, 3-T_1AM as well as the corresponding thyroacetic acid 3-TA_1 were tested concerning acute toxic effects on the used cell system MIN6, with respect to exposure time and substance concentration (Supplemental Fig. 1). We report no cytotoxic effects or impaired cell viability upon substance application *in vitro*. Cellular ATP content in the cell increased when using 1 µM 3-T_1AM concentration.

3-T_1AM transport into MIN6 cells – gene expression

To enable TH and THM interaction with mitochondria, these charged compounds need to pass the plasma membrane via THTT, but none has been specified for 3-T_1AM so far. Therefore, we screened murine pancreatic islets and MIN6 cells for matched expression of classical THTT and 3-T_1AM candidate transporter(s) described so far (Ianculescu, Friesema, Visser et al., 2010,Ianculescu, Giacomini and Scanlan, 2009) (Fig. 2). MIN6 cells show high expression of monocarboxylate transporter 10 (*Mct10)* and L-type amino acid transporter 1 (*Lat1*) (Fig. 2A). Lat2 is expressed to a lower extent and Mct8 is completely absent. Low expression of Mct8 and high expression of Lat1 was found in murine pancreatic islets, too (Fig. 2B). In contrast expression of Lat2 and especially Mct10 is more pronounced in islets compared with MIN6. Three (Slc7a1, SlcO3a1, Slc31a1) of the 3-T_1AM candidate transporters are expressed in mouse as well as human pancreas (Kounnis, Ioachim, Svoboda et al., 2011). Although described *in silico*, we found no SlcO3a1 expression in MIN6 cells and murine pancreatic islets. In contrast, Slc7a1 and Slc31a1 were highest expressed in MIN6 cells (Fig. 3A) as well as abundantly expressed in murine pancreatic islets (Fig. 2B).

Metabolism of 3-T_1AM in MIN6 cells – gene expression

In addition to THTT, cellular expression pattern of metabolizing enzymes was evaluated (Fig. 2), as 3-T_1AM can be deiodinated (Dio1-3) and oxidized (MaoA/B). In both, MIN6 cells and pancreatic islets, expression of *Dio3* and *Dio2* was absent (Fig. 2). *Dio1* shows low expression levels in MIN6

in contrast to pancreatic islets with higher *Dio1* expression. MIN6 cells as well as pancreatic islets express exclusively *MaoB*, whereas *MaoA* was not detectable.

3-T_1AM is taken up by MIN6 cells

MIN6 cells were incubated with 100 nM 3-T_1AM ± 200 µM iproniazid for up to 30 min, followed by 3-T_1AM analysis in cell lysates by LC-MS/MS (Fig. 3). Endogenous 3-T_1AM was not detectable (time point 0 min) in MIN6 cells (Fig. 3A). From 2.5 min of exposure on, 3-T_1AM was clearly detectable in the cell lysate (about 20 pmol 3-T_1AM / mg protein) and increases over time up to 60 pmol / mg protein. In the group treated with iproniazid, we observed less 3-T_1AM in the cell lysate over time, giving a first evidence towards 3-T_1AM metabolism.

Intracellular metabolism of 3-T_1AM in MIN6 cells

As Dio and Mao are exclusively intracellular enzymes, metabolism of 3-T_1AM provides evidence for 3-T_1AM uptake. The use of enzymatic inhibitors in addition helps to distinguish effects caused by 3-T_1AM or its metabolites. MIN6 cell have low endogenous concentrations of 3-TA_1 (2 pmol/mg protein, Fig. 3B). During 3-T_1AM (100 nM) exposure, 3-TA_1 concentration in the cell increases significantly already after 2.5 min, peaks after 10 min (6 pmol /mg protein) and remains elevated over the investigated time period. Preincubation with iproniazid prevents Mao-dependent metabolism of 3-T_1AM. 3-TA_1 was the only metabolite detectable by LC-MS/MS, although we analysed for e.g. T_0AM or TA_0, too.

Export of the 3-T_1AM-metabolite 3-TA_1

Cellular 3-T_1AM concentration was significantly lower in the group with iproniazid-pretreatment (Fig. 3B). This discrepancy cannot be completely explained by intracellular accumulation of the 3-T_1AM metabolite 3-TA_1 (steady state of 4 pmol/mg protein, excluding peak after 10 min, Fig. 3B). We therefore analysed the cell culture supernatant with LC-MS/MS to control for 3-T_1AM (Fig. 3C) and 3-TA_1 export (Fig. 3D). We analysed the export of 3-T_1AM in cell culture supernatants during exposure time. Although 3-T_1AM content increases fast in the cell lysate, concomitant decrease in

the supernatant is less present (Fig. 3C). 3-TA_1 was already detectable in cell culture supernatants from MIN6 cells after 5min of exposure with 100 nM 3-T_1AM (Fig. 3D). Following the time course, 3-TA_1 concentration increases linearly over time in the supernatants (from 5 up to 20 pmol/mg protein). 3-TA_1 concentration in the supernatant approximately compensates for decrease in intracellular 3-T_1AM content. Cell culture supernatants from MIN6 cells preincubated with iproniazid, are free from 3-TA_1 throughout the time course. This indicates a preferential unidirectional import of 3-T_1AM accompanied by preferential export of its oxidation product 3-TA_1 in MIN6 cells.

3-T_1AM reduces glucose-stimulated insulin secretion

Using the MIN6 cell line, we tested the hypothesis that moderate 3-T_1AM concentrations modulate GSIS directly in the β-cell (Fig. 4). Simultaneous exposure of 10-100 nM 3-T_1AM together with 2.8 mM glucose significantly decreased cellular insulin output with respect to the corresponding solvent control (Fig. 4A). Potassium chloride (KCl, 30 mM) was used as positive control because it acts as a non-specific membrane stimulator. Additional preincubation with iproniazid reversed the 3-T_1AM-dependent inhibition of GSIS (Fig. 4B). These data suggest, that intracellular metabolism to 3-TA_1 contributes to 3-T_1AM-induced hypoinsulinemia, because it is less pronounced under Mao-inhibition.

3-T_1AM and 3-TA_1 affect mitochondrial activity

As 3-T_1AM is imported by MIN6 cells, metabolized to 3-TA_1 and decreased GSIS, we analysed the potential interaction of 3-T_1AM and 3-TA_1 with mitochondria as intracellular target in order to unravel one potential signaling pathway linking THM and insulin secretion (Fig 5.). We hypothesized as a mechanism that 3-T_1AM and/or to a lesser extent its metabolite 3-TA_1 decrease mitochondrial ATP-production, resulting in lower insulin secretion. We used the Seahorse Bioanalyzer and stimulated intact MIN6 cells with moderate concentrations of 3-T_1AM and 3-TA_1. As depicted in Fig. 5A 10 nM 3-T_1AM significantly reduced mitochondrial ATP-production, with increasing potency at higher concentrations. Under control conditions, cellular mitochondria are able to convert 70 % of the supplied glucose energy to ATP but nM 3-T_1AM exposure decreases oxygen consumption rate

(OCR) to 10 – 20 %. This is due to an increased proton leak, whereas non-mitochondrial respiration is unchanged (data not shown). 3-TA_1 (1 µM) also significantly reduced mitochondrial ATP-production (Fig. 5B). Although unlikely, a contribution of 3-TA_1 to 3-T_1AM effects cannot be excluded.

We analysed in the same series of experiments if in addition maximal cellular respiration is disturbed by acute incubation with increasing concentrations of 3-T_1AM or 3-TA_1. We tested, if under conditions of complete substrate supply for mitochondria, cellular respiration can be increased over basal respiration. Under control conditions MIN6 cells were able to increase their respiration by almost 100 % (Fig. 5C). With exposure to increasing nM concentrations of 3-T_1AM the spare respiratory capacity over basal decreased (60 – 20 %). In contrast, 3-TA_1 had no significant effect probably due to higher variation within and between the performed analyses, while an inhibitory effect at 1 µM 3-TA_1 is detectable (Fig. 5D).

Using 100 nM 3-T_1AM, mitochondrial ATP-production as well as cellular respiratory spare capacity decreased, suggesting one possible signalling way of 3-T_1AM in pancreatic β-cells resulting in decreased insulin output, while its metabolite 3-TA_1 showed only minor potency.

Discussion

Cellular transport and glucoregulatory effects of 3-T_1AM and its metabolites

Using an *in vitro* cell culture system we analysed uptake, metabolism and action of 3-T_1AM in murine pancreatic β-cells (MIN6). LC-MS/MS analysis demonstrated that 3-T_1AM is taken up into MIN6 cells as indicated by its intracellular metabolism to 3-TA_1. This metabolic step was blocked by iproniazid, an unspecific aminoxidase inhibitor. We screened for *MaoA* and *MaoB* mRNA expression, but only *MaoB* transcripts were detectable using qPCR suggesting that 3-T_1AM is converted to 3-TA_1 via *MaoB*. Whether 3-T_1AM passes the plasma membrane through a transporter or via another mechanism, e.g. Apo-B100 mediated endocytosis (Roy, Placzek and Scanlan, 2012), remains to be clarified. So far, only a few approaches to identify 3-T_1AM transporters at the plasma membrane are documented. Using a large-scale RNA interference a systematic screen of the whole SLC-family of transporter genes was done (Ianculescu et al., 2009). Ianculescu et al. identified eight

different candidate-transporters (SLC7A1, SLC16A7, SLC17A5, SLCO3A1, SLCO4A1, SLC9A2, SLC31A1 and SLC43A3), sharing thyronamine specificity and transport-dependency on Na^+/Cl^- ions as well as pH. At least three of them are expressed in the human and murine pancreas: SLCO3A1, SLC7A1 und SLC31A1 (Peters, Thaete, Wolf et al., 2003,Krokowski, Han, Saikia et al., 2013). Our data indicate that the latter two are present in murine pancreatic islets and MIN6 cells and highly expressed with respect to all other studied transporters. Single and combined knock down of the three mentioned transporter in the same cell system followed by studies on 3-T_1AM uptake and intracellular metabolism might provide insight into their contribution enabling or limiting 3-T_1AM effects on GSIS. A combined knock down of transporters will be necessary, because the probability is high that 3-T_1AM is transported by different THTT exhibiting distinct functional characteristics, as already discussed in the literature (Ianculescu et al., 2010,Ianculescu et al., 2009). A saturable cell-specific mechanism for 3-T_1AM uptake appears critical for the maintenance of physiological control (Janssen and Janssen, 2017). T_3 and T_4 are transported via different THTT like MCT8, MCT10, LAT1 and LAT2 with tissue-specific expression profiles (Friesema, Ganguly, Abdalla et al., 2003,Friesema, Jansen, Jachtenberg et al., 2008,Visser, Friesema and Visser, 2011,Wirth, Schweizer and Kohrle, 2014).

Inhibition of transporter dependent thyronine upake by 3-T_1AM

T_3 itself is an essential growth factor, regulating cellular metabolism, resulting in enhanced cellular growth and differentiation. Type I diabetes mellitus (DM) induces destruction of pancreatic β-cells through apoptotic processes. Falzacappa *et al.* tested in pancreatic β-cell lines (rat and human) as well as in primary pancreatic rat islets if T_3 has an anti-apoptotic effect on β-cells in culture (Verga Falzacappa, Panacchia, Bucci et al., 2006,Verga Falzacappa, Patriarca, Bucci et al., 2009,Verga Falzacappa, Mangialardo, Raffa et al., 2010). They showed that T_3 stimulates cell proliferation, while cell number increases under this treatment and cell viability is enhanced. This occurs through interaction of T_3 with TRβ1 subsequently leading to regulation of pro- and anti-apoptotic factors. These effects are dependent on PI3K and pAkt308. Their studies revealed, that uptake of TH in pancreatic β-cells supports survival and maintenance of function. Therefore, an assured transport of

TH, across the plasma membrane via THTT is essential. Ianculescu *et al.* demonstrated in 2010, that micromolar 3-T_1AM concentrations inhibit the transport of T_3 and T_4 via OATP1A2 (IC_{50} = 0.27 and 2.1 µM), transport of T_4 through OATP1C1 (IC50 = 4.8 µM), as well as T_3- and T_4-uptake via MCT8 (IC_{50} = 95.0 and 31.0 µM) but mechanism of inhibition was not studied in detail. This data may be an indirect hint for 3-T_1AM transport via these THTT, although mere inhibition of T_4 and T_3 transport by 3-T_1AM is a second possibility. It was not examined so far if 3-T_1AM restricts β-cell function by blocking TH transporter (Ianculescu et al., 2010). It is only known that 3-T_1AM does not impact MCT10 transport properties (Ianculescu et al., 2010). Even though the Mct10 expression in isolated islets and MIN6 cells is really high with respect to Mct8, Mct10 is a likely transporter for thyronines, but excluding 3-T_1AM. Moreover, none of the analysed OATP is expressed in the healthy human pancreas. Expression of OATP1A2, 1B3 and 1C1 only increase in expression in the state of pancreas carcinoma. Oatp1c1 is not expressed at all in MIN6 cells. Slco4c1 and 1a5 are not or only to a low extent expressed in MIN6 cells and murine Langerhans islets. Hence, the contribution of these transporters to 3-T_1AM uptake in the studied cell culture model is unlikely. Mct8 shows low expression levels in MIN6 cells and murine pancreatic islets, too. 3-T_1AM transport via this THTT is unlikely, because the published IC_{50}-value (Kinne, Kleinau, Hoefig et al., 2010) is far above the concentration range used in our experiments. As 3-T_1AM uptake experiments reported here were performed in absence of serum which, contains ApoB100, a lipoprotein with high avidity for 3-T_1AM (Roy et al., 2012), such an THTT-independent contribution to 3T1AM uptake by LDL-receptor mediated endocytosis, intercellular metabolism and cellular action in Min6 cells can be neglected.

Intracellular metabolism and consequences for insulin secretion

3-T_1AM is rapidly taken up and subsequently metabolized in MIN6 cells via MaoB to yield 3-TA_1. Generation of 3-TA_1 can be suppressed by Mao inhibitors. Our data indicates that 3-TA_1 is not accumulated but exported from MIN6 cells in concentration- and time-dependent manner. In addition to export, the intracellular 3-TA_1 concentration remains over time in an elevated steady state. Exposure of MIN6 cells to nanomolar 3-T_1AM concentrations decreases GSIS. Uptake of 3-

399 T_1AM has been described for other target cell types (Saba et al., 2010,Ghelardoni et al.,
400 2014,Agretti, De Marco, Russo et al., 2011,Schanze, Jacobi, Rijntjes et al., 2017). Schanze et al.
401 reported 3-T_1AM uptake and its metabolism by PTU-sensitive Dio1 and iproniazid-sensitive MAO for
402 the rat thyroid cell line FRTL, which release 3-TA_1 and T_0AM into the culture medium (Schanze et
403 al., 2017). Exposure of these cells to 3-T1AM alters expression of thyroid-relevant genes and Ca^{2+}
404 signalling (Schanze et al., 2017). Ghelardoni *et al.* demonstrated 3-T_1AM uptake into HepG2 cells
405 and its metabolism to 3-TA_1 (Ghelardoni et al., 2014). After its formation 3-TA_1 also accumulates in
406 the cell supernatant, indicating its exchange across the hepatocyte plasma membrane. In MIN6
407 cells, in contrast to HepG2 cells, 3-TA_1 concentration transiently reaches an elevated intracellular
408 steady state concentration while at the same time being exported leading to increasing
409 concentrations in the cell culture conditioned medium, reaching concentrations manifold above the
410 intracellular concentration. Therefore, contribution of 3-TA1 to modulation of insulin secretion cannot
411 be excluded. Ghelardoni et al. reported on the 3-T_1AM stimulated gluconeogenesis in HepG2 cells,
412 which is prevented in presence of a Mao-inhibitor (Ghelardoni et al., 2014). Direct incubation with 3-
413 TA_1 alone was not able to restore the gluconeogenic effect, but no further details were provided. If
414 applied i.c.v., 3-T_1AM-dependent hyperglycemia, reduction of serum fT_3-levels, improvement of
415 learning capacity as well as reduced pain-susceptibility for hot stimuli were all abolished or changed
416 in outcome by pre-treatment with the unspecific Mao-inhibitor chlorgyline (*i.p.*). However, 3-TA_1 itself
417 was not directly tested in comparison. Similar Mao-related changes in outcome were observed in
418 our study presented here. GSIS as well as mitochondrial ATP-turnover were significantly decreased
419 upon 3-T_1AM treatment. The Mao-inhibitor iproniazid, diminishes GSIS suppression by 3-T_1AM. In
420 contrast, direct incubation of MIN6 cells with 3-TA_1 was less effective on mitochondrial ATP-
421 turnover. We conclude from this data that a direct effect of 3-TA_1 cannot be excluded, but is
422 dependent on its intracellular formation from 3-T_1AM via MaoB, whereas these effects are less
423 pronounced like in heart and thyroid (Schanze et al., 2017,Rutigliano and Zucchi, 2017) or even
424 absent if MIN6 cells are directly exposed to 3-TA_1. One reason may be, that import of 3-TA_1 is
425 inefficient, while 3-T_1AM may serve as directional shuttle system to pass the plasma membrane that
426 is however permeable for 3-TA_1 export. Intracellular conversion to 3-TA_1 may serve as local

regulation mechanism of intracellular 3-T_1AM concentration, necessary if 3-T_1AM import is not strictly regulated or unspecific and thus resulting in increased intracellular accumulation of 3-T_1AM. 3-TA_1 itself is predominantly exported and accumulates in the cell culture medium. Further investigations have to be performed to clarify the underlying context.

In contrast to HepG2 cells 3-T_1AM is metabolized preferably to T_0AM in perfused hepatocytes. It is unknown so far if a comparable discrepancy can also be expected for MIN6 cells compared to primary murine Langerhans islets. Referring to the literature we are only aware of the fact that 3-T_1AM reaches the pancreas through the circulation (Chiellini, Erba, Carnicelli et al., 2012), but no further metabolites from 3-T_1AM could be analysed *in vivo*. Due to comparable enzymatic expression pattern (*Dio* and *Mao*), we assume that the cell line and the murine pancreas share similarities in metabolism.

Mechanism of 3-T_1AM action – binding to plasma membrane vs. cellular uptake

Following the 3-T_1AM uptake into MIN6 cells we observed reduced mitochondrial ATP-production and decreased GSIS. Although uptake was proven by intracellular metabolism of 3-T_1AM to 3-TA_1, 3-T_1AM interaction with GPCR, TRP channels or TAAR at the plasma membrane cannot be excluded. But uptake and intracellular interaction with mitochondria provide a new way of direct glucohomeostatic regulation in pancreatic β-cells. Regard et al. already investigated in 2007 the role of 3-T_1AM in GPCR mediated insulin secretion (Regard et al., 2007). They demonstrated that 3-T_1AM modulates insulin secretion negatively via G_i-coupled $Adra_{2\alpha}$ as well as positively via G_s-coupled TAAR1 receptor. They demonstrated that a single high dose application of 50 mg/kg i.p., as already described by Scanlan et al., induces hyperglycemia and hypoinsulinemia. Direct inhibition of G_i-coupled signaling pathways in pancreatic β-cells prevented the 3-T_1AM induced effects on glucohomeostasis *in vivo*. *In vitro* experiments using isolated primary murine and human pancreatic islets showed that the high concentration of 10 µM 3-T_1AM inhibits GSIS. This effect was reversed by suppressing $G_{i/o}$-coupled signalling pathways. As it was known before that 3-T_1AM binds Taar1 resulting in elevated cAMP levels they suggested an interaction with G_i signalling (Scanlan et al., 2004). But this approach failed to be an explanation as the effect persisted in TAAR1 knockout mice

455 (Regard et al., 2007), resulting in the conclusion that there has to be another GPCR, coupling $G_{i/0}$
456 and being activated by binding of 3-T_1AM. In addition to TAAR1, $Adra_{2\alpha}$ is highly expressed in
457 pancreatic islets that is known to couple to $G_{i/0}$. 3-T_1AM has structural relations to catecholamines
458 as main activating compounds. Membrane-binding studies showed, that 3-T_1AM binds as good as
459 or even better to $Adra_{2\alpha}$ in comparison to epinephrine (Dinter, Muhlhaus, Jacobi et al., 2015).
460 Besides this, 3-T_1AM application in $Adra_{2a}$ null-mice did not induce hyperglycemia. Central
461 regulation of hyperglycemia via $Adra_{2\alpha}$-expression in neurons was also excluded. Recently,
462 experiments in transfected HEK cells revealed 3-T_1AM induced $G_{i/0}$ activation at $Adra2_{2\alpha}$ and
463 reduction of the norepinephrine-induced MAPK activation (Dinter et al., 2015). This data supports
464 an agonistic effect of 3-T_1AM on the $Adra2_{2\alpha}$ mediated $G_{i/0}$ pathway, while antagonizing MAPK
465 activation after stimulation with norepinephrine. In this study the *in vivo* administration of 3-T_1AM
466 over six days at the lower daily dose of 5 mg/kg did not alter glucose homeostasis in mice (Dinter et
467 al., 2015), indicating different outcome with respect to the dose and duration of treatment.
468 Together, this data give a strong hint that an acute high dose of 3-T_1AM induces hyperglycemia
469 directly in the pancreas dependent on interaction with $Adra_{2\alpha}$ coupled to $G_{i/0}$. As we demonstrated,
470 3-T_1AM uptake and rapid metabolism by pancreatic β-cells *in vitro* we consider cellular uptake as a
471 second molecular event to initiate intracellular 3-T_1AM action independent from interactions with
472 receptors at the plasma membrane. In addition to the data presented here we are aware that
473 plasma membrane initiated signaling pathways resulting in hypoinsulinemia cannot be excluded by
474 our approach. Clear results may be generated using MIN6 cells with induced $Adra_{2\alpha}$ knock down,
475 even though we consider that activation of this receptor needs pharmacologic micromolar 3-T_1AM
476 concentrations much higher than those nanomolar concentrations resulting in decreased GSIS and
477 OCR in our experiments.
478
479 **Mitochondria are 3-T_1AM target structures**
480 We present data demonstrating 3-T_1AM interference with mitochondrial energy metabolism in the
481 nanomolar concentration range resulting in concentration-dependent decrease in ATP-turnover.
482 Comparable or micromolar concentrations of 3-TA_1 had minor effects on mitochondrial action. Thus,

3-T_1AM is modulating mitochondrial energy production more efficiently. 3-T_1AM has already been described in literature as non-competitive inhibitor of F_0/F_1-ATP synthase (Venditti et al., 2011). Venditti et al. used isolated liver mitochondria from hypothyroid rats to demonstrate that 3-T_1AM decreases oxygen consumption and increases H_2O_2 production. Two interaction sites with different affinities were identified for 3-T_1AM in the respiratory chain. Kinetic analysis using sub-mitochondrial particles, soluble ATPase, membrane-integrated F_0/F_1-ATPase as well as 3-T_1AM treated permeabilized cardiomyocytes (H9c2) showed that low submicromolar 3-T_1AM concentrations have a positive effect on mitochondrial energy production. In contrast, high 3-T_1AM concentrations favoured binding to a low affinity-site of F_0/F_1-ATP synthase. Enzyme activity was blocked and ATP turnover was reduced. We showed in intact MIN6 cells, that 3-T_1AM concentration-dependently inhibits ATP-turnover compared to a DMSO solvent control. As 3-T_1AM accumulates in MIN6 cells over time, we propose that these concentrations are sufficiently high to decrease mitochondrial ATP-production favouring the idea that 3-T_1AM may interact with the above described F_0/F_1-ATP synthase low-affinity binding site in this target cell.

Human relevance

So far only one preliminary human study is documented describing the association between 3-T_1AM serum concentration and DM (Galli et al., 2012). This study reported on a significant relation between 3-T_1AM and fT_4, tT_4, fT_3 as well as tT_3 serum concentrations (n=42). In a subpopulation (n=18) they tested for DM type II. Endogenous 3-T_1AM serum concentrations were significantly elevated in seven patients with diagnosed DM type II compared to non-diabetic patients. In addition, 3-T_1AM was significantly associated with HbA_1c as well as fasting blood sugar. Authors concluded from the data, that individuals with elevated 3-T_1AM values in the circulation tend to be hyperglycemic in combination with impaired insulin secretion. For sure, the studied population was too small for a generalized statement. Such associations have to be addressed in a bigger cohort with DM type II to confirm the hypothesis. However, results from this human study harmonize with local effects of 3-T_1AM on pancreatic β-cells in our study where exposure of MIN6 cells to 10-100

510 nM 3-T_1AM significantly reduced GSIS. This effect may support hyperglycemia, as described in the
511 preliminary human study.
512
513 In summary, we present novel data on uptake, intracellular metabolism and action of 3-T_1AM in a
514 pancreatic β-cell model resulting in decreased GSIS at low nanomolar concentrations of this TH
515 metabolite due to a mitochondrial mechanism.
516
517 **Acknowledgments**
518 We like to thank Dr. Daniel Rathmann for LC-MS/MS analysis as well as Anja Fischbach and Kristin
519 Fischer for technical assistance with extractions for LC-MS/MS and Seahorse Bioanalyzer
520 measurements. In addition the authors want to thank Dr. Oliver Kluth for preparing and providing
521 cDNA from murine pancreatic islets as well as Dr. Mette Stoedter for transfer of knowledge about
522 handling MIN6 cells.
523
524 **Funding**
525 This work is supported by grants from the Deutsche Forschungsgemeinschaft (DFG SPP1629
526 Thyroid Trans Act, HO 5096/2-1 to C.S.H.; KO 922/16-2 and 922/17-2 to J.K).

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695

696 **Figure legends**

697 **Figure 1: Metabolism of 3-Iodothyronamine (3-T_1AM) to generate the corresponding 3-**
698 **iodothyroacetic acid (3-TA_1).** The alanine side chain of 3-T_1AM is modified by monoamine oxidase
699 (MAO) and semicarbazid-sensitive aminoxidase (SSAO) to generate 3-iodothyroacetic acid 3-TA_1.

700

701 **Figure 2: MIN6 cell line and murine primary pancreatic islets express a comparable enzyme-**
702 **and transporter pattern.** Expression profile of enzymes and transporters involved in handling of TH
703 by MIN6 cells (A) compared to murine primary pancreatic islets (B). RNA was isolated from
704 technical triplicates of three consecutive MIN6-passages. RNA from murine primary pancreatic islets
705 was combined, generating three individual pools from different animals each. qRT-PCR data is
706 presented as Mean ± SEM.

707

708 **Figure 3: Time course of 3-T_1AM uptake (A) and 3-TA1 formation (B) by murine MIN6**
709 **pancreatic cells and their concentration profile in conditioned media (C,D) in absence (●) or**
710 **presence (■) of the Mao inhibitor iproniazid.** 3-TA_1 remains at an elevated intracellular steady
711 state concentration, peaking after 10 min, following 3-T_1AM application (B). 3-T_1AM declines in
712 conditioned medium over time (C). 3-TA_1 as intracellular metabolite of 3-T_1AM is exported from
713 MIN6 cells in increasing concentrations over time (D). MIN6 cells were incubated with 100 nM 3-
714 T_1AM for 0 - 30 min with (■) or without (●) 200 µM iproniazid as Mao-inhibitor (A-D). Extracted cell
715 lysates (A & B) and cell culture supernatants (C & D) were analysed for their 3-T_1AM and 3-TA_1
716 concentration using LC-MS/MS. Data are generated from three individual experiments using
717 triplicates each, normalized to protein content and presented as Mean ± SEM. Statistics: two-sided
718 t-test per time point (A-C); ** P<0.01, * P<0.05, ns = not significant. Data-fitting: one-sided-specific
719 binding (A & C).

720

721 **Figure 4: 3-T_1AM impairs insulin secretion in MIN6 cells dependent on Mao activity. 3-T_1AM-**
722 **dependent changes in glucose-stimulated insulin secretion (GSIS).** MIN6 cells were incubated

723 with 100 nM 3-T_1AM and 2.8 mM glucose as well as without (A) or with 200 µM iproniazid as Mao-
724 inhibitor (B). Insulin secretion was measured in cell culture supernatants. Data are normalized to
725 cellular protein content. Data is generated from three individual experiments using triplicates each
726 and presented as relative insulin secretion based on solvent-treated control (Mean ± SEM). 3-T_1AM
727 and 3-TA_1 both contribute to lowered insulin secretion, because Mao-inhibition reduces the
728 aforementioned effect (not shown). Statistics: One-way ANOVA with Bonferroni post test; ***
729 P<0.001, ** P<0.01 * P<0.05, ns = not significant.
730
731 **Figure 5: 3-T_1AM and to a lower extent 3-TA_1 decrease mitochondrial ATP-production (A & B)**
732 **as well as maximal respiration over basal (spare capacity, C & D) in MIN6 cells.** MIN6 cells
733 were acutely treated with 3-T_1AM (A,C) or 3-TA_1 (B,D) with increasing concentrations as well as
734 different mitochondrial inhibitors to determine mitochondrial functions. Normalization: OCR-values
735 for oligomycin (A & B) as well as FCCP (C & D) are presented as % basal respiration, excluding
736 non-mitochondrial respiration. Data is generated from three individual experiments with consecutive
737 passages of MIN6 cells. Each substance incubation contains six technical replicates (Mean ± SEM).
738 Statistic: One-way ANOVA (Kruskal-Wallis test) with Dunns Post Test; *** P< 0.001, ** P< 0.01.
739
740 **Fig. 6: Schematic overview of 3-T_1AM effects in pancreatic MIN6 cells as β-cell model.** 3-T_1AM
741 passes transporter-dependent the plasma membrane, is intracellular metabolized via MaoB to TA_1,
742 which in return is released from the cell via a not further specified exporter. 3-T_1AM and partly 3-TA_1
743 can reach target structures at the mitochondria resulting in decreased ATP-turnover. The signalling
744 metabolite ATP reduces 3-T_1AM-dependently the insulin secretion upon glucose stimulation.

745 **Supplement**

746 **Material and methods**

747 **Toxicity test**

748 MIN6 cells were seeded at a density of 20,000 cells / 200 µl (96 well) in cell culture medium. Then
749 culture medium was removed and replaced with FBS-free culture medium for ON incubation. At day
750 three culture medium was removed and replaced by KRBH, containing the indicated concentrations
751 of 3-T_1AM, 3-TA_1 or the corresponding DMSO control concentrations. After 2 h 10 % Vol 3-(4,5-
752 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 1 mg/ml) was added and incubation
753 was continued for additional 2 h at 37 °C 5 % CO_2. Substance incubation and MTT exposure were
754 stopped by aspiration of medium followed by addition of 0.1 ml lysis-buffer (10 % sodium dodecyl
755 sulphate, 0.6 % acetic acid in DMSO). Cells were lysed for 5 min at room temperature (RT) and
756 homogenized by 5 min shaking. Photometric measurement was performed at 595 nm.

757

758 **ATP production – cell viability test**

759 MIN6 cells were seeded at a density of 10,000 cells/0.1 ml (96 well) in cell culture medium. Culture
760 medium was removed and replaced with FBS free culture medium for ON incubation. At day three,
761 medium was removed and replaced by KRBH, containing the indicated concentrations of 3-T_1AM, 3-
762 TA_1 or the corresponding DMSO solvent concentrations. Substance exposure was stopped after 2 h
763 by adding CellTiter Glo substrate (0.1 ml), followed by 2 min shaking to induce cell lysis and 10 min
764 incubation at RT to stabilize the luminescence signal. Luminescence was read in a luminometer and
765 ATP production analysis was done as recommended by the supplier (Cell Titer Glo, Promega,
766 Madison, USA).

767

768 **Results**

769 **Cytotoxicity and cell viability upon 3-T_1AM and 3-TA_1 application in pharmacological dose**

770 Before starting exposure experiments, 3-T_1AM as well as the corresponding 3-iodothyroacetic acid
771 3-TA_1 were tested concerning acute toxic effects on the used cell system MIN6, with respect to
772 exposure time and substance concentration.

3-T_1AM and 3-TA_1 were administered acutely in the concentration range of 10 nM – 10 µM. Because effects on glucose homeostasis in animal models occurred fast within hours, we conducted experiments with a readout after 0.5-4.0 h. Increasing concentrations of 3-T_1AM and 3-TA_1 showed after 4 h of exposure the same relative cytotoxicity as the DMSO-control using the MTT cytotoxicity test (Suppl. Fig. 1A & 1B). Because this NADH/NADPH dependent assay is less sensitive, referring especially to the acute exposure time, a second ATP-dependent cell viability assay was performed. It was expected, that rising substance concentration may decrease the portion of living cells. In contrast, high concentrations of 3-T_1AM (1-10 µM) doubled cellular ATP-content (Suppl. Fig. 1C). Lower concentrations (10-100 nM) had no effect, as cell viability was comparable to the DMSO control. Exposure with 3-TA_1 showed a 20 % increase of ATP in this cell system with 10 µM only (Suppl. Fig. 1D). ATP is one of the signaling metabolites supporting insulin secretion (Maechler, 2003). Based on our data we suggested that 3-T_1AM concentrations above 100 nM might cause hyperinsulinemia due to high ATP production or accumulation, contrasting *in vivo* data from animal models. We conclude from these first data to use substance concentrations not higher than 100 nM 3-T_1AM in this respective cell line.

Figures legend

Supplemental Fig. 1: 3-T_1AM increases intracellular ATP content at high pharmacological dosages, but has no cytotoxic effects. Cytotoxicity (A & B) and cell viability (C & D) of MIN6 cells during 3-T_1AM (A & C) or 3-TA_1 (B & D) incubation. MIN6 cells were incubated with increasing concentrations of either 3-T_1AM or 3-TA_1 (10 nM-10 µM) to test acute substance toxicity. Data is presented as Mean ± SEM. Mean OD_{595} of cell wells treated with DMSO as control is represented as 100 %. 3-T_1AM and 3-TA_1 treated cells are shown as % with respect to the control. Statistics: One-way ANOVA (Kruskal-Wallis test); * $P<0.05$, *** $P<0.001$. Data are results from two (cell viability test) or three (cell toxicity test) independent experiments using six wells per group and per substance concentration. Solvent control and substance incubations contain the same DMSO concentration.

Supplemental Tables

Supplemental Table 1: Overview of investigated genes and corresponding primers to detect transcripts of THTT and metabolizing enzymes in MIN6 cell line as well as murine primary pancreatic islets.

	Gene	Sequence	Amplicon length	Name
TH transporter	**Slc16a10 fwd**	GCCCCATCGTGAGTGTCTTC	182 bp	*Mct10*
	Slc16a10 rev	GACCAGTGACGGCTGGTAGG		
	Slc16a2 fwd	CCTCGCTATGGGCATGATCT	202 bp	*Mct8*
	Slc16a2 rev	TGGTTGAAAGGCGAATGAGC		
	Slc7a8 fwd	GCCACCCGGGTTCAAGATA	189 bp	*Lat2*
	Slc7a8 rev	ATAGGCAAAGGAGCCCTGGA		
	Slc7a5 fwd	GGCCATCATCATCTCCTTGC	197 bp	*Lat1*
	Slc7a5 rev	ACCCATTGACAGAGCCGAAG		
unspecific transporter	**Slco4c1 fwd**	TGGCAAAACTTCCCAGACTCA	186 bp	*OATP4C1*
	Slco4c1 rev	AAAAATGTGGCAAATCCCGTA		
	Slco4a1 fwd	TGACCACTGACAGCCCACTG	205 bp	*OATP1*
	Slco4a1 rev	TTGCCAAAAGCTGGATTGCT		
	Slco1c1 fwd	AGTGTGGCCGGACTGACTGT	186 bp	*Oatp14*
	Slco1c1 rev	ACTCCGGCTGGAGGATTGAC		
	Slco1a5 fwd	CATCCTGACAAGTGTGCTCCAG	211 bp	*OATP3*
	Slco1a5 rev	TGCCATGTATGCAGCCTTCTT		
deiodase	**Dio1 fwd**	CACAGCCGATTTCCTCATCA	184 bp	*Dio1*
	Dio1 rev	GCTGCTCTGGTTCTGCATTG		
	Dio2 fwd	CTCCAACTGCCTCTTCCTGG	211 bp	*Dio2*
	Dio2 rev	GACGTGCACCACACTGGAAT		
	Dio3 fwd	CTACATCGAGGAAGCCCACC	203 bp	*Dio3*
	Dio3 rev	TGACGTAGAGGCGCTCAAAA		
reference gene	**hprt fwd**	GGCCAGACTTTGTTGGATTTG	144 bp	*HPRT*
	hprt rev	TGCGCTCATCTTAGGCTTTGT		
postulated 3-T_1AM transporter	**Slco3a1 fwd**	TCTTCTCGTCCCTCCTGATG	224 bp	*Slco3a1*
	Slco3a1 rev	CACAGGGTTTGAGAGCAGGT		
	Slc7a1 fwd	CTGGAGTGCGACTTTTGACG	179 bp	*Slc7a1*
	Slc7a1 rev	TGTTGACCATGGCTGACTCC		

	Slc31a1 fwd	TTGTTTTCCGGTTTGGTAATCA	178 bp	*Slc31a1*
	Slc31a1 rev	GGATGGTTCCATTTGGTCCT		
monoamine	**MaoA fwd**	GGGGCTGCTACACAGCCTAC	243	*MaoA*
oxidase	**MaoA rev**	CAAGAGCTGGAACATCCTTGG		
	MaoB fwd	ATTGCCACGCTCTTTGTGAA	237	*MaoB*
	MaoB rev	CCCTGTCTGGTCAATGTGGA		

805

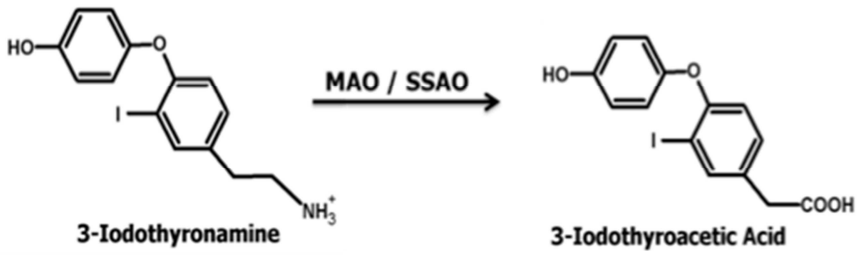
806

807 **Supplemental Table 2: Description and calculation of mitochondrial functions measured with**
808 **Seahorse XF96e Bioanalyzer.** Non-mitochondrial respiration was subtracted from individual
809 functions for calculations of reserve capacity and ATP-turnover.

Function	**Description / calculation**
Reserve capacity	= uncoupled respiration / basal respiration
ATP-turnover	= 1 – (inhibitor ATP-synthesis / basal respiration)
Basal respiration	last point of measurement before first injection
Maximal respiration	highest point of measurement after addition of uncoupling agent
Non-mitochondrial respiration	respiration after inhibition of mitochondrial electron transport

810

Figure 1



3-Iodothyronamine

3-Iodothyroacetic Acid

Figure 2

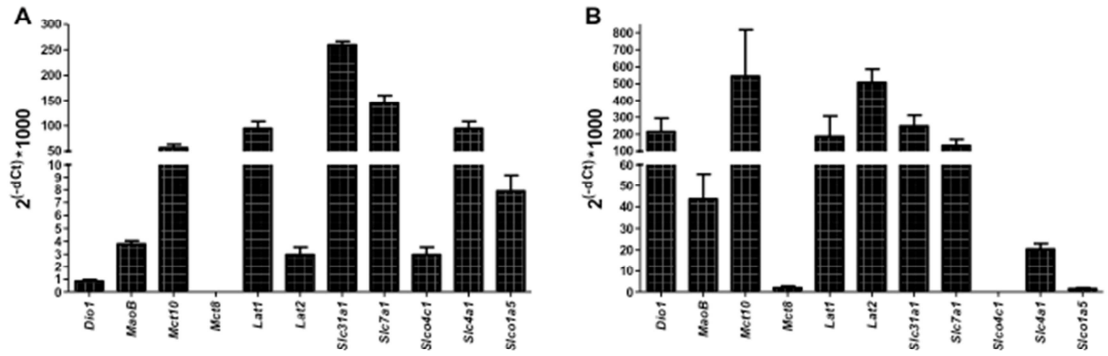


Figure 3

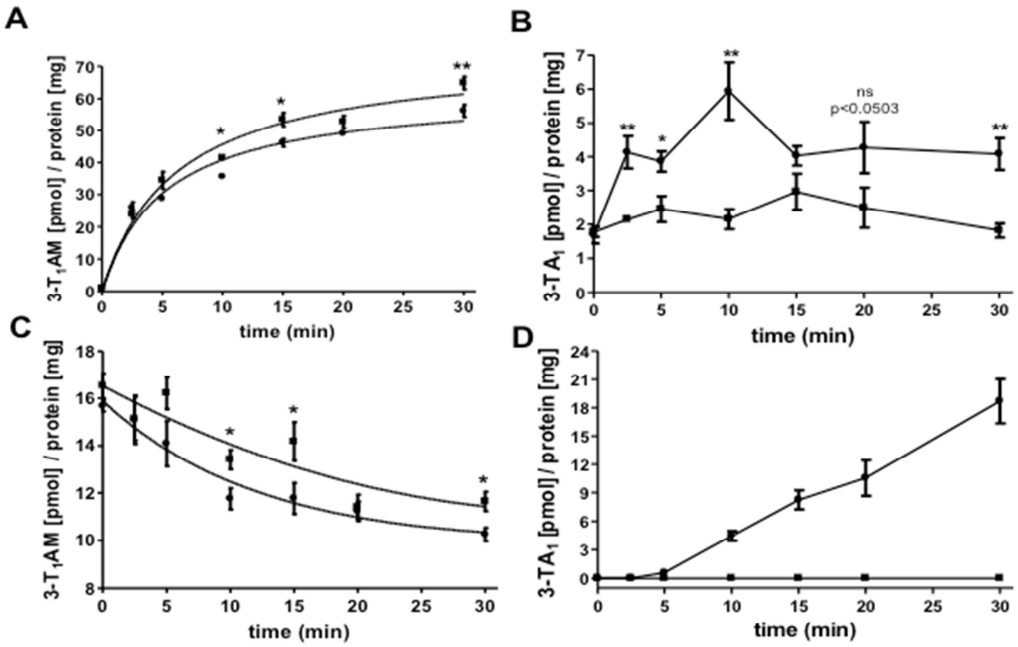
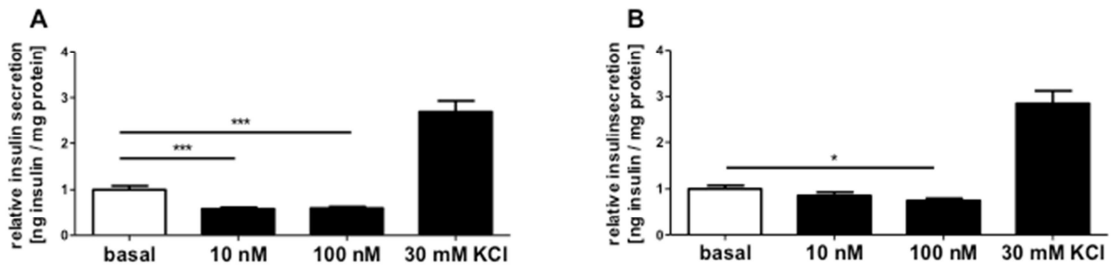


Figure 4



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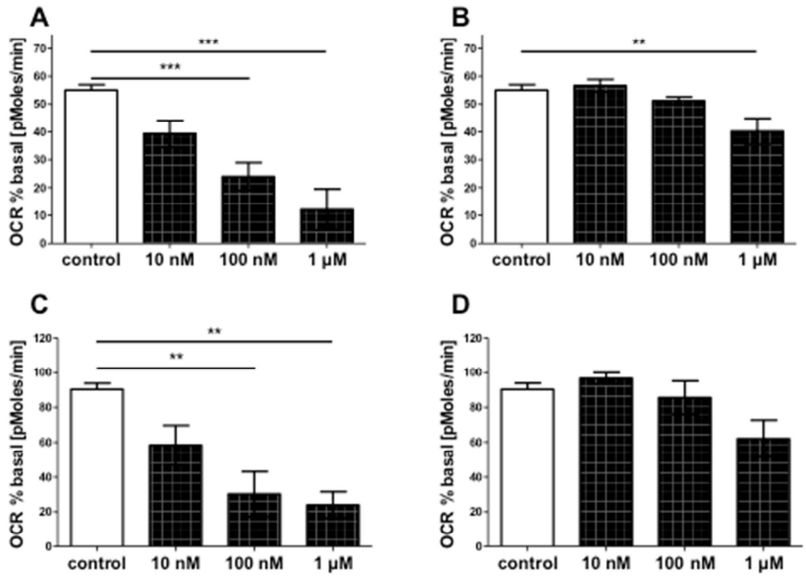
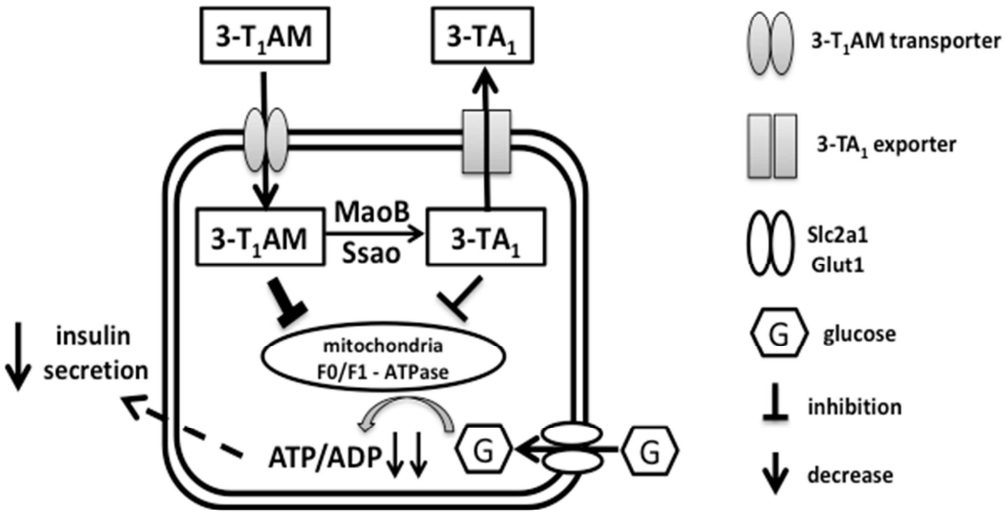


Figure 6



Highlights

- 3-T_1AM influences glucoregulatory processes *in vivo* and *in vitro*
- 3-T_1AM decreases GSIS due to a mitochondrial mechanism
- Min6 cells transport and metabolize 3-T_1AM
- Pancreatic MIN6 cells were used as suitable *in vitro* β-cell model