Uptake and metabolic effects of 3-iodothyronamine in hepatocytes

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Abstract

3-Iodothyronamine (T_1AM) is an endogenous relative of thyroid hormone with profound metabolic effects. In different experimental models, T_1AM increased blood glucose, and it is not clear whether this effect is entirely accounted by changes in insulin and/or glucagone secretion. Thus, in the present work, we investigated the uptake of T_1AM by hepatocytes, which was compared with the uptake of thyroid hormones, and the effects of T_1AM on hepatic glucose and ketone body production. Two different experimental models were used: HepG2 cells and perfused rat liver. Thyronines and thyronamines (T0AMs) were significantly taken up by hepatocytes. In HepG2 cells exposed to 1 μM T_1AM, at the steady state, the cellular concentration of T_1AM exceeded the medium concentration by six- to eightfold. Similar accumulation occurred with 3,5,3′-triiodothyronine and thyroxine. Liver experiments confirmed significant T_1AM uptake. T_1AM was partly catabolized and the major catabolites were 3-iodothyroacetic acid (TA1) (in HepG2 cells) and T0AM (in liver). In both preparations, infusion with 1 μM T_1AM produced a significant increase in glucose production, if adequate gluconeogenetic substrates were provided. This effect was dampened at higher concentration (10 μM) or in the presence of the amine oxidase inhibitor iproniazid, while TA1 was ineffective, suggesting that T_1AM may have a direct gluconeogenetic effect. Ketone body release was significantly increased in liver, while variable results were obtained in HepG2 cells incubated with gluconeogenetic substrates. These findings are consistent with the stimulation of fatty acid catabolism, and a shift of pyruvate toward gluconeogenesis. Notably, these effects are independent from hormonal changes and might have physiological and pathophysiological importance.

Key Words
- thyronamines
- hepatocytes
- metabolism
- glucose production

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Introduction

3-Iodothyronamine (T_1AM) is an endogenous relative of thyroid hormone, which is able to produce acute functional effects (Scanlan *et al.* 2004, Piehl *et al.* 2011). Recent advances in the quantitative analysis of T_1AM using methods based on HPLC coupled to tandem mass spectrometry (MS/MS) have shown that T_1AM can be detected in blood and tissues derived from rodents and humans, and that in several rat tissues, including heart, liver, kidney, skeletal muscle, and stomach, T_1AM concentration is greater than 3,5,3′-triiodothyronine (T_3) and thyroxine (T_4) concentration (Saba *et al.* 2010, Galli *et al.* 2012). Although structural similarities between T_4 and T_1AM have led to the hypothesis that T_1AM is produced in the peripheral tissues by T_4 deiodination and decarboxylation, the biosynthetic origin of T_1AM remains an open issue. Serum analysis of thyroid cancer

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patients treated with T_4 (Hoefig *et al.* 2011) provided evidence for extrathyroidal T_1AM production, and limited T_1AM formation was observed in cardiomyocytes incubated with T_3 (Saba *et al.* 2010). On the other hand, a recent study has revealed that T_1AM is not an extrathyroidal metabolite of T_4, and is produced by a process that requires the same biosynthetic factors necessary for T_4 synthesis, namely the sodium–iodide symporter and thyroperoxidase (Hackenmueller *et al.* 2012).

The molecular target(s) of T_1AM are largely unknown. *In vitro* studies provided evidence that T_1AM can activate with high-affinity G protein-coupled receptors, including trace amine-associated receptor 1 (Scanlan *et al.* 2004, Zucchi *et al.* 2006) and possibly α2-adrenoceptors (Regard *et al.* 2007). In addition to these receptors, T_1AM also interacts with plasma membrane and vesicular biogenic amine transporters (Snead *et al.* 2007) as well as different mitochondrial targets (Venditti *et al.* 2011, Cumero *et al.* 2012).

Administration of exogenous T_1AM and other thyronamines (T0AMs) produced functional effects that showed a rapid onset and were often opposite to those induced by T_3 (Liggett 2004, Weatherman 2007). In rodents, i.p. T_1AM injection rapidly induced hypothermia, decreased cardiac function and decreased the respiratory quotient, suggesting a shift from primarily carbohydrate to predominantly lipid utilization (Scanlan *et al.* 2004, Chiellini *et al.* 2007, Braulke *et al.* 2008). Recent results from NMR-based metabolomics and breath studies have shown that chronic T_1AM exposure induced a rapid increase in lipid mobilization, followed after a few days by increased protein breakdown (Haviland *et al.* 2013). T_1AM-treated mice showed continued reduction in body weight, independent of food consumption, and after T_1AM withdrawal they regained only 1.8% of the lost weight in the following 2 weeks. Intracerebroventricular (i.c.v.) injection of T_1AM modified hormone secretion, food intake, and memory acquisition (Dhillo *et al.* 2009, Klieverik *et al.* 2009, Manni *et al.* 2012, 2013).

T_1AM effects were not linearly related to the dosage and depended on the animal species and administration route (Dhillo *et al.* 2009, Klieverik *et al.* 2009, Manni *et al.* 2012). The response to T_1AM may also be affected by its complex metabolism, which includes oxidative deamination (to 3-iodothyroacetic acid, TA1), deiodination (to T0AM), sulfation (to *O*-sulfonate-T_1AM), acetylation (to *N*-acetyl-T_1AM), and glucuronidation (to T_1AM-glucuronide) (Wood *et al.* 2009, Hackenmueller & Scanlan 2012).

Among its metabolic effects, T_1AM has been reported to produce hyperglycemia (Regard *et al.* 2007, Klieverik *et al.* 2009). Notably, hyperglycemia occurs after administration of relatively low doses of exogenous T_1AM, producing changes in tissue concentration of about one order of magnitude (Manni *et al.* 2013). In addition a clinical investigation performed in a small series of patients revealed that serum T_1AM concentration was significantly correlated with HbAlc, and significantly increased in a subgroup of diabetic patients (Galli *et al.* 2012). Therefore, the effects of T_1AM on glucose metabolism might have physiological and pathophysiological relevance. The mechanism of this effect is largely unknown. As hyperglycemia was observed after i.c.v. injection, it was originally attributed to central regulation of endocrine function, namely stimulation of glucagon secretion and/or inhibition of insulin secretion (Klieverik *et al.* 2009). However, it was recently observed that even after i.c.v. injection, a significant increase in plasma T_1AM was produced (Manni *et al.* 2012), and the possible occurrence of peripheral effects on glucose metabolism has been suggested. Because of the central role of liver in glucose homeostasis, the aim of the present work was to establish whether T_1AM affects glucose metabolism in perfused liver and in a hepatocellular carcinoma cell line (HepG2 cells). An additional aim was to determine the uptake and metabolism of T_1AM in the same preparations.

Materials and methods

Chemicals

T_1AM, T0AM, TA1, TA0, and their deuterated derivatives used for HPLC–MS/MS were synthesized as described elsewhere (Hart *et al.* 2006, Miyakawa & Scanlan 2006, Wood *et al* 2009). Unless otherwise specified, all other reagents were from Sigma–Aldrich. Solvents for HPLC–MS/MS measurements were HPLC grade, and the other chemicals were reagent grade.

Cell culture and treatment

Human hepatocellular carcinoma cells (HepG2), obtained from American Type Culture Collection (Manassas, VA, USA), were cultured in DMEM supplemented with 10% (v/v) fetal bovine serum, 1 mM pyruvate, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a humidified atmosphere containing 5% CO_2 and subcultured before confluence.

The experiments aimed at evaluating hormone uptake and metabolism were carried out as described previously, with minor modifications (Saba *et al.* 2010). Briefly, cells

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were seeded into 24-well plates (8.5×10^4 cells/well) and grown to 80% confluence. At the start of each experiment, the culture medium was removed and replaced with 0.5 ml of Krebs–Ringer medium (118 mM NaCl, 25 mM $NaHCO_3$, 4.5 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 1.5 mM $CaCl_2$, 1 g/l glucose, pH 7.4) and preincubated in a humidified atmosphere of 5% CO_2 at 37 °C for 30 min. Incubation was initiated by quickly replacing the pre-incubation medium with 0.5 ml Krebs–Ringer buffer, containing one of the following compounds: T_1AM, T_3, T_4 at 1 μM concentration. For each of the tested compound, a 1 mM stock solution in DMSO was previously prepared. The plate was returned to a humidified atmosphere of 5% CO_2 at 37 °C, the medium was removed from each well at specific time points (5 min to 24 h), centrifuged (11 600 ***g*** for 2–3 min) to eliminate detached cells, and analyzed by HPLC–MS/MS analysis. The cell plates were then frozen for 24–48 h and lysed in 0.1 ml 0.1 M NaOH. After pH neutralization (0.01 ml 1.0 N HCl), the cell lysates were diluted with Krebs–Ringer to a final volume of 0.5 ml, centrifuged at 5000 ***g*** for 10 min, and used for HPLC–MS/MS analysis. As assessed in previous experiments, where cells were removed from the well by scraping in the presence of ice-cold PBS followed by centrifugation at 5000 ***g*** for 10 min, the packed cell volume was on the order of 0.02 ml. Therefore, we can assume that during cell lysates preparation (0.5 ml, final volume), the cellular content in the final sample was diluted by about 25-fold.

Similar experiments were carried out to examine the effect of amine oxidase inhibitor, iproniazid, on T_1AM metabolism. The cells were preincubated for 30 min with 0.5 ml Krebs–Ringer buffer supplemented with iproniazid (10 mM stock solution in DMSO to a final concentration of 100 μM) before adding T_1AM.

To assess glucose and ketone body release, HepG2 were seeded into six-well plates (5×10^5 cells/well) and grown to 80% of confluence with standard medium. Before treatment, the cells were washed twice with PBS and then exposed for 4 h to exogenous T_1AM (0.1, 0.5, 1, 5, and 10 μM) or TA1 (0.5 and 1 μM) in 1 ml DMEM base, glucose- and phenol red-free, containing 100 U/ml penicillin, 100 μg/ml streptomycin, and 4 mM L-glutamine, supplemented with 2 mM sodium pyruvate and 20 mM sodium lactate (glucose production buffer; Yoon *et al.* 2001) at 37 °C in 5% CO_2. In some experiments, the amine oxidase inhibitor iproniazide (100 μM) was also included. Control cells were incubated with DMEM containing DMSO (10–20 μl/well).

To assess free fatty acid (FFA) release, HepG2 were exposed to exogenous 1 μM T_1AM for four or 24 h in DMEM base (glucose- and phenol red-free), containing 100 U/ml penicillin, 100 μg/ml streptomycin, and 4 mM glutamine, supplemented with 1 g/l glucose and 1 mM sodium pyruvate.

To induce steatosis, HepG2 were exposed to exogenous lipids as described (Di Nunzio *et al.* 2011, Yao *et al.* 2011). Cells were incubated with 1 μM T_1AM in DMEM base (glucose- and phenol red-free) containing 100 U/ml penicillin, 100 μg/ml streptomycin, 4 mM glutamine, 1 g/l glucose, 1 mM sodium pyruvate, and supplemented with 1 mM of FFA mixture (2:1 ratio of oleate and palmitate) in 1% BSA for 24 h (stock solution of 30 mM FFA was conveniently diluted to 1 mM in culture medium). In each experimental protocol, control cells were incubated with supplemented DMEM-containing DMSO.

Cell culture medium was then collected and glucose, ketone bodies (acetoacetate and 3-hydroxybutyrate), and fatty acid levels were evaluated.

Rat liver perfusion

This investigation conforms to the Declaration of Helsinki and the Guiding Principles in the Care and Use of Animals. The project was approved by the Animal Care and Use Committee of the University of Pisa.

Male Wistar rats (275–300 g body weight), fed a standard diet, were anesthetized with a mixture of ether and air and the livers were perfused *in situ* with glucose-free Krebs–Ringer buffer or glucose production buffer equilibrated with a mixture of O_2 (95%) and CO_2 (5%) at a constant flow rate of 10 ml/min. The fluid was introduced through a cannula inserted into the portal vein, while a second cannula inserted into the inferior cava vein was used to collect the effluent perfusate. After 10 min washout period and 10 min perfusion with Krebs–Ringer buffer alone, T_1AM (1 μM or 50 nM) was added to the non-recirculating buffer, and the liver was perfused for another 40 min, followed by 10 min of perfusion with Krebs–Ringer buffer alone (Mario *et al.* 2009). In control experiments, liver was perfused for 60 min with Krebs–Ringer buffer alone. During the experiment, samples of the perfusion buffer were collected at 5 min intervals, and at the end of the experiments the liver was removed and frozen at −80 °C.

In the experiments to evaluate hormone up-take and metabolism, isolated rat liver was perfused with 50 nM or 1 μM T_4, T_3, or T_1AM in Krebs–Ringer buffer, as described earlier, and tissue concentration was determined after

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60 min. HPLC–MS/MS analysis that allowed the simultaneous detection of T_3, T_4, T_1AM, and its putative metabolites, namely T0AM, TA0, and TA1, was carried out as described elsewhere (Saba *et al*. 2010).

Metabolite assays

Glucose concentration was assessed in 100 µl of medium or perfusate with a colorimetric glucose assay kit (Sigma–Aldrich). Ketone bodies were evaluated in the medium or perfusate by enzymatic methods as elsewhere described (Alberti & Hockaday 1972). Briefly, acetoacetate was determined in a reaction mix containing 0.05 mM K_2HPO_4 (pH 7), 0.02% NADH, and 0.25 U/ml 3-hydroxybutyrate dehydrogenase. The mixture was incubated for 45 min at 30 °C and absorbance was read at 340 nm to evaluate NADH reduction. 3-Hydroxybutyrate was measured in a reaction mix containing 0.03 M Tris–HCl (pH 8.5), 2% hydrazine, 0.06% EDTA, 0.02% NAD, and 0.25 U/ml 3-hydroxybutyrate dehydrogenase. The mixture was incubated for 45 min at 30 °C and absorbance was read at 340 nm to evaluate NADH production.

Fatty acid concentration was determined in the cell culture medium with a FFA quantification kit (BioVision Research Product, Malpitas, CA, USA) upon extraction. Fatty acids were extracted by adding 200 µl of dichloromethane to 850 µl of medium. The mixture was vortexed and spinned at 10 000 ***g*** for 5 min. The organic phase was collected and vacuum dried for 1 h to remove dichloromethane. Dried lipids were then dissolved in 50 µl of assay buffer provided by the kit and used for the assay.

Metabolite concentrations were referred to the total protein content (Bradford 1976) of whole-cell lysates (in HepG2 experiments) or to wet tissue weight (in liver experiments).

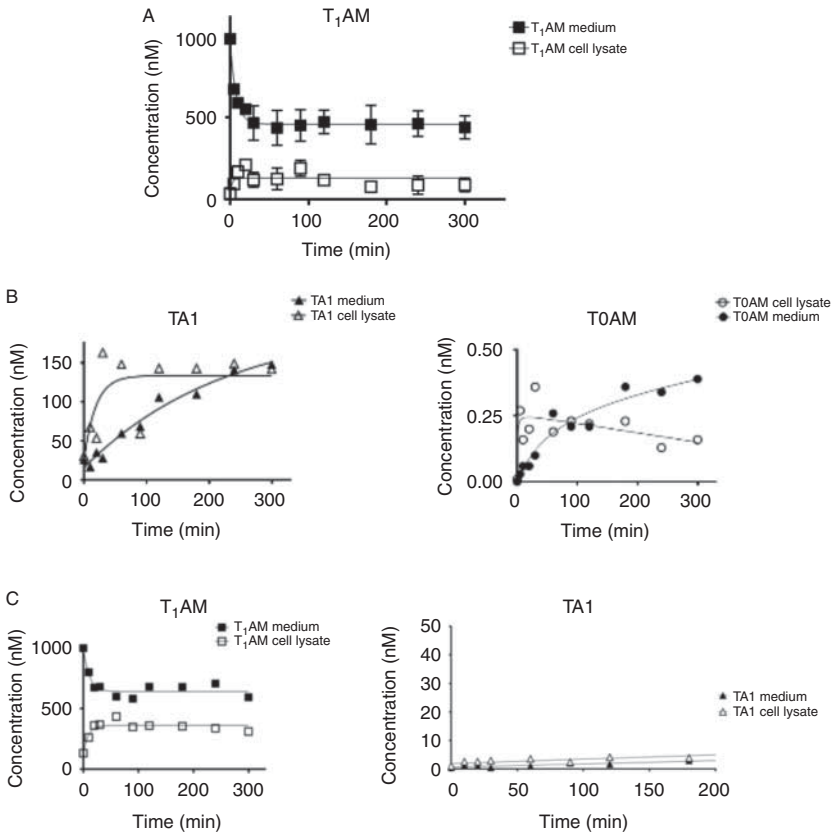

Figure 1

T_1AM up-take and metabolism in HepG2 cells (subjected to 20–25 passages *in vitro*): (A) results of T_1AM up-take in HepG2 cell during 6 h of incubation with 1 µM T_1AM (mean±S.E.M. of nine biological replicates). (B) Catabolite production during incubation with 1 µM T_1AM: representative results of T0AM and TA1 assay; (C) representative results of T_1AM and TA1 assay in experiments carried out in the presence of 100 µM iproniazid. Assays were performed at different time in the incubation medium and in the cell lysate. During preparation, the lysate was diluted ~20- to 25-fold to a final volume of 500 µl.

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Statistical analysis

Results are expressed as the mean ± S.E.M. Differences between groups were analyzed by ANOVA or, when only two groups were involved, by unpaired *t*-test. Regression analysis was performed by linear, exponential, or hyperbolic models, as detailed in the description of each experiment. The threshold of statistical significance was set at $P<0.05$. GraphPad Prism version 6.0 for Windows (GraphPad Software, San Diego, CA, USA) was used for data processing and statistical analysis.

Results

T_1AM uptake and catabolism in HepG2 cells and in perfused rat liver

When cells were exposed to 1 μM T_1AM, its concentration in the incubation medium decreased exponentially, while lysate T_1AM increased, reaching a steady state after about 10 min (Fig. 1A). In these experiments, incubation medium volume was 500 μl, while the original volume of cell lysate was on the order of 20–25 μl, and the latter was diluted to a final volume of 500 μl to facilitate processing and assay. Steady-state concentrations were close to 500 and 150 nM in the incubation medium and in diluted cell lysate respectively (Fig. 1A). Taking into account the dilution factor, the actual steady-state cellular concentration can be estimated to average about 3–4 μM, exceeding medium concentration by over six- to eight-fold. The overall recovery of T_1AM was on the order of 65–70% (500 pmol were added and the final amounts detected in incubation medium and in cell lysate averaged ~250 and 75 pmol respectively).

T_1AM adhesion to cell culture plates, as determined by carrying out the experiment in the absence of cells, was limited, accounting only for about 10% decrease in T_1AM concentration. Additional experiments were also carried out at 4 °C, and the rate constant of the exponential decay decreased from 0.052 ± 0.006/min to 0.025 ± 0.004/min ($P<0.01$), suggesting that active biochemical process are involved in this phenomenon.

Medium and lysate were also assayed for T_1AM catabolites. TA1 accumulated over time both in lysate and in medium, reaching a concentration of about 140 nM in both compartments after 240 min (due to lysate dilution, this corresponds to an estimated cellular concentration of 2.8–3.5 μM). T0AM was detected at very low concentration (<0.5 nM) (Fig. 1B), while TA0 was not revealed (data not shown). Overall catabolite production accounted for about 30% of T_1AM administration.

Experiments were repeated in the presence of the amine oxidase inhibitor iproniazid, and in these conditions T_1AM recovery averaged 99%, because after 240 min incubation medium and lysate concentrations averaged 700 and 300 nM, respectively, while TA1 production was almost abolished (Fig. 1C) and T0AM was not significantly modified (data not shown).

By comparison, similar experiments were performed by incubating HepG2 cells with T_3 or T_4. As observed with T_1AM, lysate concentrations increased while medium concentrations decreased exponentially, until a steady state was obtained after about 120 min. At the steady state, medium concentrations averaged 651 ± 73 and 413 ± 91 nM for T_3 and T_4 respectively. The corresponding lysate concentrations were 135 ± 59 and 343 ± 137 nM respectively. The overall recovery was close to 80% for T_3 and T_4.

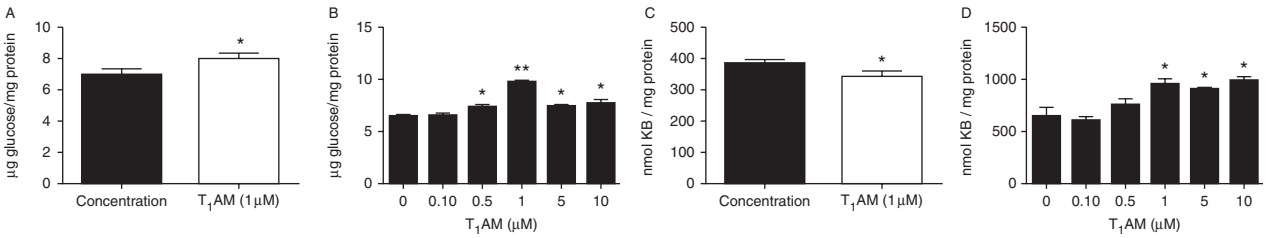

Figure 2
Glucose and ketone body (KB) production in HepG2 cell cultures that were incubated for 4 h in glucose production buffer as described in Materials and methods. Glucose or KB concentration was assayed in the medium. Results are expressed as mean ± S.E.M. and are normalized to the total cell protein content determined in cell lysates. (A) Glucose concentration after incubation with 1 μM T_1AM ($n=9$ per group). (B) A dose–response curve was obtained using 0.1, 0.5, 1, 5, 10 nmol/ml of T_1AM, corresponding to 0.1, 0.5, 1, 5, and 10 μM concentrations ($n=3$ in each case). (C and D) KB production: (A and C) 1 μM T_1AM administered to cells which underwent >30 *in vitro* passages ($n=9$), while in (B and D) a dose–response curve ($n=3$) was obtained in cells which underwent <20 *in vitro* passages. See text for further details. *$P<0.05$, **$P<0.01$ by ANOVA or unpaired *t*-test, as appropriate.

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T_1AM, T_3, and T_4 uptake were also investigated in perfused rat liver. After 40 min of perfusion with 50 nM or 1 μM T_1AM, tissue concentration averaged 87.2±22.0 and 1013.8±312.5 pmol/g, respectively, vs a control value of 7.1±4.2 pmol/g. Among putative catabolites, only T0AM was detected in the liver homogenate at concentrations approximately one order of magnitude lower than T_1AM. In this model, T_1AM uptake was lower than T_3 and T_4 uptake, because after 40 min of perfusion with 50 nM T_3 or T_4 their tissue concentrations averaged 853.6 and 1531.2 pmol/g, respectively, vs control values of 3.9 and 15.5 pmol/g. If perfusate T_3 and T_4 concentrations were raised to 1 μM, tissue concentrations after 40 min of perfusion averaged 2.14 and 3.90 μmol/g respectively. In the latter experiments, the assay of T_3 and T_4 in the perfusion buffer confirmed that over 90% of infused hormones were taken up and stored in the tissue.

Notably, perfusion with T_3 or T_4 was not associated with significant increase in tissue T_1AM, nor was perfusion with T_1AM was not associated with any significant change in tissue T_3 or T_4 (data not shown).

Metabolite assays

In HepG2 cells cultured in glucose production buffer (Fig. 2A), incubation with 1 μM T_1AM induced ~25% increase in glucose production ($P<0.01$). In other experiments, the dose dependence of this effect was investigated (Fig. 2B): the estimated EC_{50} was 0.84 μM, but at 5 or 10 μM T_1AM the stimulation of glucose production decreased, yielding a bell-shaped dose–response curve.

More complex findings were obtained with regard to ketone body production. Baseline ketone body production and the response to T_1AM apparently depended on the number of passages performed *in vitro*. Using cells which underwent >30 passages *in vitro*, treatment with 1 μM T_1AM produced a slight (11%) but significant decrease in ketone body release (Fig. 2C), chiefly accounted for by decreased acetoacetate release (252.4±10.5 vs 286.8±7.2 nmol/mg protein, $P<0.05$), while no significant effect was detected using 0.1 or 10 μM T_1AM (data not shown). However, when we took care to use cells that underwent no more than 20 passages, we observed that baseline ketone body production was higher (on the order of 630 nmol/mg proteins), and under these conditions T_1AM produced a dose-dependent stimulation of ketone body release, with EC_{50} on the order of 1.12 μM (Fig. 2D).

In the presence of 100 μM iproniazid, the increase in glucose production induced by 1 μM T_1AM was dampened (9%) and did not reach the threshold of statistical significance (5.99±0.17 vs 6.53±0.31 μg/mg protein in cell lysate, P=NS). We also tested the effect of TA1, the major T_1AM catabolite, and, at 500 nM or 1 μM concentration, it did not affect either glucose or ketone body release. Notably, cellular uptake of TA1 was substantially lower than that observed with T_1AM (15 vs 60%).

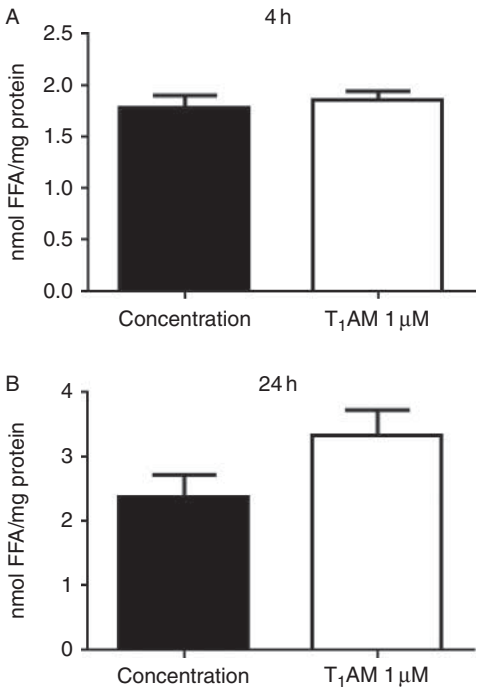

Figure 3
Free fatty acid (FFA) production in HepG2 cell cultures that were incubated for 4 h (A) or 24 h (B) in DMEM medium as described in Materials and methods. Results are expressed as mean±S.E.M. of nine biological replicates and are normalized to the total cell protein content determined in cell lysates. Differences between groups were not statistically significant (unpaired *t*-test).

In the presence of exogenous glucose, T_1AM did not produce any change in fatty acid production after 4 or 24 h of incubation (Fig. 3A and B), although at the later time point there was a slight increase that did not reach the threshold of statistical significance (P=0.088). Under conditions promoting steatosis, i.e. with supplementation of exogenous glucose and fatty acid mixture, after 24 h T_1AM did not affect FFA concentration, while glucose concentration was increased ($P<0.01$), suggesting increased production and/or decreased consumption (Fig. 4A and B).

Liver perfusion with glucose production buffer in the presence of 1 μM T_1AM (Fig. 5) also showed a significant increase in glucose production (Fig. 5A, $P<0.05$) that was associated with increased ketone body release (Fig. 5B, $P<0.01$), largely accounted for by increased

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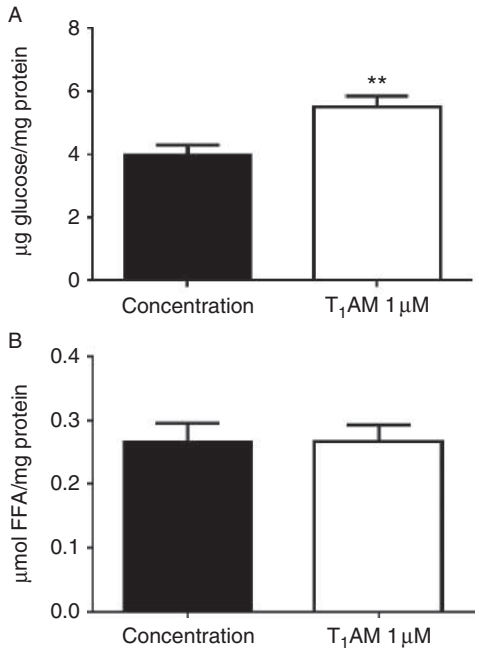

Figure 4
Glucose (A) and fatty acids (B) concentration in HepG2 cell cultures after incubation for 24 h in conditions promoting steatosis, i.e. DMEM medium containing 1 mM free fatty acids 1 mM and 1 g/l glucose. Results are expressed as mean ± S.E.M. of nine biological replicates and are normalized to the total cell protein content determined in cell lysates. $^{**}P<0.01$ by unpaired *t*-test.

3-hydroxybutyrate release (246.0 ± 21.04 vs 153.3 ± 11.81 nmol/min per g, $P<0.01$).

Infusion with Krebs–Ringer buffer did not produce any significant change in metabolite release (glucose: 0.24 ± 0.17 vs 0.29 ± 0.02 mg/min per g, P=NS; ketone bodies 40.1 ± 4.9 vs 35.1 ± 3.0 nmol/min per g, n=8–9). A few experiments (n=2) were also carried out in livers obtained from 4 h fasted rats, and a 26% increase in the release of ketone bodies was observed (231 ± 22 vs 175 ± 25 nmol/min per g), while glucose release was not affected (0.28 ± 0.06 vs 0.28 ± 0.05 mg/min per g).

Discussion

In this investigation, we observed that T_1AM, such as T_3 and T_4, can be taken up and accumulated in hepatocytes. In HepG2 cell culture, these compounds were quickly absorbed because their presence was detected in cell lysate after a few minutes of infusion. At the steady state, estimated cellular concentration exceeded medium concentration by about six- to eightfold. These results are in agreement with previous observations performed in cardiomyocytes (Saba *et al.* 2010) and in FRTL-5 thyroid cells (Agretti *et al.* 2011), and they are consistent with the results obtained in perfused liver, where tissue concentration was higher than perfusate concentration. In this model, the ratio between tissue and perfusate concentration was lower than the lysate:medium ratio observed in cell cultures, but it should be considered that T_1AM is likely to be taken up and catabolized by vascular and interstitial cells, and that it undergoes biliary excretion (Chiellini *et al.* 2012).

In HepG2 cells, T_1AM recovery was on the order of 70%. Most of the balance was accounted for by oxidative deamination, yielding the thyroacetic derivative TA1 that was detectable in cell lysate and medium within a few minutes. Although D1 deiodinase has been reported to be expressed in HepG2 cells (Jakobs *et al.* 2002), T0AM production was minimal, probably because of the absence of D3 deiodinase and/or of essential cofactors for deiodination or decarboxylation, while it was greater in perfused liver, where it appears to be more relevant than oxidative deamination. We cannot, however, exclude that additional metabolic pathways may be active, particularly conjugation to sulfate and glucuronide (Hackenmueller & Scanlan 2012), because the corresponding derivatives could not be tested by the present method.

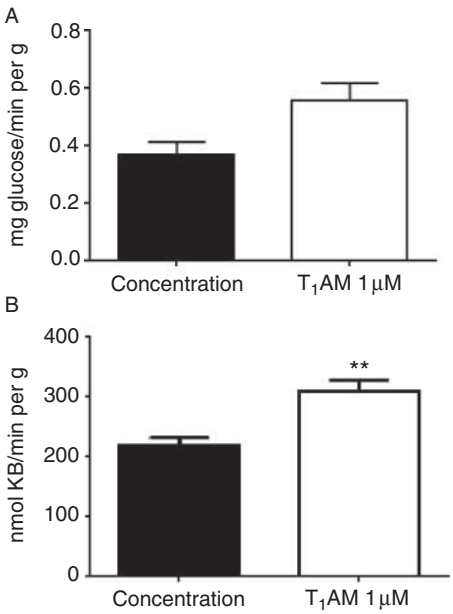

Figure 5
Glucose (A) and ketone body (KB) (B) release by livers perfused with 1 µM T_1AM in glucose production buffer. Glucose and KB concentrations were determined in the perfusate every 5 min for a total period of 40 min, and the average release rate was calculated, as described in Materials and methods. Results are expressed as mean ± S.E.M. of the three experiments and are normalized to tissue wet weight. $^{**}P<0.01$ by unpaired *t*-test.

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It should be stressed that about 50% of administered T_1AM, T_3, or T_4, was detected in cell lysate after 2 h of infusion. As a consequence at the steady state the estimated cellular concentration of T_3, T_4, and T_1AM was significantly higher than medium concentration. This suggests the existence of specific binding sites and/or transport pathways for T_1AM and thyroid hormones.

The molecular mechanism by which T0AMs are taken up is still controversial. Eight potential transporters belonging to the solute carrier family have been tentatively identified (Ianculescu *et al*. 2009), although their affinity and sodium-independence did not seem to be consistent with the characteristics of T_1AM uptake (Saba *et al*. 2010). Recently it has been reported that T_1AM is largely bound to apolipoprotein B-100 (apo-B100), the protein component of LDLs (Roy *et al*. 2012). This could be a potential vehicle to transport T_1AM and other T0AMs into cells which expressed the receptor for LDL, even though this is unlikely to contribute to our results, because in our experiments the standard growth medium supplemented with fetal bovine serum was replaced with other media, namely Krebs buffer or glucose production buffer, which do not contain lipoproteins. In any case, the kinetics of T_1AM removal from the incubation medium was significantly lower at 4 °C, suggesting the presence of active transport mechanisms.

The primary aim of this work was to establish whether T_1AM may directly stimulate hepatic glucose production. If adequate substrates for gluconeogenesis were provided, T_1AM increased glucose production in two different experimental models, namely cultured HepG2 cells and *in situ* perfused rat liver. So consistent results were obtained in different experimental models, which also investigate different time scales, because the former was a subchronic and the latter an acute model. We conclude that modulation of hepatic metabolism may contribute to the hyperglycemic effect reported after administration of exogenous T_1AM (Regard *et al*. 2007, Klieverik *et al*. 2009, Manni *et al*. 2012), which had previously been attributed only to modulation of insulin and/or glucagone secretion.

In HepG2 cells, the EC_{50} for the effect on gluconeogenesis was in the submicromolar range, and in perfused liver experiments a significant effect was observed with infusion of 1 μM T_1AM, resulting in tissue concentration on the order of 1000 pmol/g. It is not easy to discuss the potential physiological relevance of these observations, as there is still some uncertainty about the physiological levels of T_1AM. In humans, using an immunological assay (Hoefig *et al*. 2011), the plasma concentrations were reported on the order of 66 nM, while plasma T_1AM concentration measured by a mass spectrometry-based assay was about 200-fold lower (Galli *et al*. 2012). As T_1AM binds with high affinity to apo-B100 (Roy *et al*. 2012), it has been hypothesized that these different techniques estimate total and free T_1AM respectively. However, T_1AM is known to be concentrated in tissues, as discussed above, and we have previously reported that its liver content in rat *in vivo* was on the order of 90 pmol/g (Saba *et al*. 2010). If we rely on this value, then we may suggest that infusion of exogenous 1 μM T_1AM raised liver concentration to a value that was one order of magnitude greater than the physiological concentration, after the depletion presumably occurring during prolonged perfusion with T_1AM-free buffer (after over 60 min of perfusion 'control' values were close to 1 pmol/g). Further experiments will be necessary to clarify these important issues.

The effect of T_1AM on gluconeogenesis may show a bell-shaped dose–response curve, because the response of HepG2 was reduced at the highest concentration tested (10 μM). This is not surprising, because other functional effects of T_1AM, particularly on feeding and behavior, are also biphasic (Dhillo *et al*. 2009, Hettinger *et al*. 2010, Manni *et al*. 2012). Consistent with this interpretation, the response to 1 μM T_1AM decreased in the presence of iproniazid, which inhibits its deamination to TA1, while we did not obtain any significant response after administration of exogenous TA1, whose cellular uptake was very low.

Another effect attributed to T_1AM is the stimulation of lipid catabolism, with a shift from glucose to fatty acid as energy source (Braulke *et al*. 2008, Haviland *et al*. 2013). We observed a similar effect in the perfused liver model, because T_1AM induced an increase in the release of ketone bodies. The results obtained in the HepG2 model were more complex, and in a subset of cells showing low values of baseline ketone body production T_1AM reduced ketogenesis.

The different timing of these two experimental models might partly account for this discrepancy, because in the conscious mouse T_1AM-induced stimulation of lipid catabolism was also time dependent (Haviland *et al*. 2013). Alternatively, it might be suggested that energy production by HepG2 cells, which are known to have limited ketogenic capability (Vilà-Brau *et al*. 2011), may be significantly dependent on pyruvate and amino acid catabolism, so that pyruvate shift toward gluconeogenesis would cause less acetyl-CoA to be available for ketogenesis. Notably, in HepG2 cells no significant effect was apparent on fatty acid production.

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In conclusion, we observed that T_1AM is actively accumulated in hepatocytes, where at concentrations in the micromolar range it is able to stimulate gluconeogenesis and to stimulate ketogenesis, provided that adequate energy substrates are available. These effects are independent from hormonal changes and might have physiological and pathophysiological importance. It would also been interesting to investigate whether T_1AM may contribute to the metabolic effects usually attributed to thyroid hormone and/or to the metabolic abnormalities observed in hypothyroidism, a condition in which tissue and plasma T_1AM levels have been reported to be decreased (Galli *et al*. 2012, Hackenmueller *et al*. 2012).

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

S G and G C designed and carried out cell culture experiments and metabolic assays. S F performed the *ex vivo* experiments on liver. A S carried out mass spectrometry measurements. R Z designed and supervised the experimental work and wrote the manuscript.

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