

Published in final edited form as:

*FEBS Lett.* 2011 February 4; 585(3): 539–544. doi:10.1016/j.febslet.2010.12.044.

## The chemical chaperones tauroursodeoxycholic and 4-phenylbutyric acid accelerate thyroid hormone activation and energy expenditure

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### Abstract

Exposure of cell lines endogenously expressing the thyroid hormone activating enzyme type 2 deiodinase (D2) to the chemical chaperones tauroursodeoxycholic acid (TUDCA) or 4-phenylbutyric acid (4-PBA) increases D2 expression, activity and T3 production. In brown adipocytes, TUDCA or 4-PBA induced T3-dependent genes and oxygen consumption (~2-fold), an effect partially lost in D2 knockout cells. In wild type, but not in D2 knockout mice, administration of TUDCA lowered the respiratory quotient, doubled brown adipose tissue D2 activity and normalized the glucose intolerance associated with high fat feeding. Thus, D2 plays a critical role in the metabolic effects of chemical chaperones.

### 1. INTRODUCTION

Thyroid hormone accelerates energy expenditure with increases in oxygen consumption of about 4-fold during the transition from hypothyroidism to thyrotoxicosis [1,2]. Thyroid hormone is activated via the type 2 deiodinase (D2), with a number of molecules accelerating D2-mediated conversion of T4 to T3 in metabolically active tissues such as brown adipose tissue (BAT) and skeletal muscle, including bile acids, xenobiotic compounds and insulin sensitizing agents [3]. Notably, disruption in the D2 pathway decreases insulin signaling and/or energy expenditure [4,5].

D2 is an endoplasmic reticulum (ER)-resident protein with a 20-min half-life that is ubiquitinated by proteins involved in the ER-stress response [6–8]. Thus, the finding that chemical chaperones, which attenuate ER-stress, have been used in genetically obese mice to enhance insulin action and normalize metabolism [9] led us to hypothesize an involvement of D2 in this process. Chemical chaperones are small molecules known to

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stabilize protein conformation in the ER. However, with the exception of alleviating leptin resistance [10], it is not clear how 4-phenylbutyrate (4-PBA) and taurine-conjugated ursodeoxycholate (TUDCA), the reported chemical chaperones with metabolic activity, trigger their effects on metabolism [11].

We now report that both TUDCA and 4-PBA are capable of activating the D2 pathway in a number of cell models and also when administered to mice. Exposure of brown adipocytes to TUDCA or 4-PBA accelerates the production of T3 and stimulates energy expenditure via a mechanism that is D2-dependent. In addition, the use of D2 knockout (*Dio2<sup>-/-</sup>*) animals and cells indicate that important metabolic effects resulting from treatment with TUDCA also depend on the D2 pathway.

## 2. Material and Methods

### 2.1 Reagents and materials

Unless specified otherwise, all reagents were purchased from Sigma Chemical Co. (St. Louis, MO) or Calbiochem (San Diego, CA). Outer ring-labeled T4 (specific activity, 4,400 Ci/mmol) was from PerkinElmer (Boston, MA). Lipofectamine-Plus reagents were from Invitrogen (Carlsbad, CA). DMEM and RPMI-1640 were from GIBCO (Grand Island, NY).

### 2.2 Animals

Animals were maintained and experiments performed according to protocols approved by the Animal Care and Use Committee in compliance with National Institutes of Health standards. For all experiments, 8-week old male mice were used. C57BL/6J (C57) mice were obtained from Jackson Laboratories (Bar Harbor, ME). Mice homozygous for a targeted disruption of the *Dio2* gene (*Dio2<sup>-/-</sup>*) on a C57 background were described previously [12]. Unless otherwise stated, mice were fed normal chow and housed under a 12h light/12h dark cycle at 22°C. Chow pellets (#5010) were from Lab Diet, MO and high fat (TD.95121) pellets were obtained from Harlan Teklad, MA.

For calorimetric studies, mice were individually housed and acclimated in calorimeter cages for 2 days followed by 2 days to access baseline data and 7 days of data collection of gas exchanges and food intake as described previously [13]. Body composition was studied by dual energy x-ray absorptiometry (DEXA; Lunar Piximus, Janesville, WI). For the procedure, mice were anesthetized using Avertin (Tribromoethanol) 0.3–0.6mg/g and placed on the scan table for analysis [13].

### 2.3 Cells

Mesothelioma cells (MSTO-211H) and hepatocellular carcinoma cells (HepG2) were purchased from ATCC (Manassas, VA). Cells were grown and maintained in RPMI-1640 (MSTO-211H) and DMEM (HepG2), supplemented with 10% FBS and 100nM sodium selenite [14]. Brown adipocytes were differentiated in vitro from isolated primary preadipocytes, as described previously [4]. Cellular Oxygen Consumption was measured in real-time using a XF-24 Extracellular Flux Analyzer (Seahorse Bioscience Inc, Billerica, MA), as described previously [15].

### 2.4 T4 to T3 conversion and deiodinase activity

The activities of type 1 and type 2 deiodinases (D1 and D2) were measured as previously described [12]. The production of <sup>125</sup>I from outer ring-labeled T4, in intact cells was analyzed as described and validated elsewhere [15].

## 2.5 Real-time qPCR

Total RNA was extracted using TRIzol reagent (Invitrogen) and used to synthesize cDNA using SuperScript First-Strand Synthesis System (Invitrogen). The RT-qPCR was performed as previously described [15]. Cyclophilin A was used as a housekeeping gene.

## 2.6 Statistical analysis

All data were analyzed using PRISM software (GraphPad Software, Inc., San Diego, CA) and are expressed as mean  $\pm$  SEM. One-way ANOVA was used to compare more than two groups, followed by the Student-Newman-Keuls test to detect differences between groups. The Student's t test (two-tailed) was used to compare the differences between two groups;  $p < 0.05$  was used to reject the null hypothesis.

## 3. RESULTS

### 3.1 Chemical chaperones increase D2 mRNA levels, protein levels and T3 production

MSTO-211H cells, which endogenously express D2, were exposed to increasing concentrations of TUDCA or 4-PBA for 7–48h, resulting in enhanced D2 activity (2.5–3-fold) as assayed in cell sonicates (Fig. 1A–B). 4-PBA acted faster (maximum at 12h), while the TUDCA effect lagged behind and reached its maximum by 48h (Fig. 1A). Similar results were observed in the RMS-13 muscle cell line that endogenously expresses D2 as well (data not shown). The effects were highly selective for D2 given that the activity of D1, the other thyroid hormone activating deiodinase, remained unaffected in HEPG2 cells exposed to either chemical chaperone (Table 1).

To study if D2 induction leads to increased T3 production in intact cells, MSTO-211H cells were incubated with  $^{125}\text{I}$ -T4 and  $^{125}\text{I}$ -T3 production was analyzed. In this setting, it is clear that T3 production increases progressively (up to ~3-fold) in response to exposure to either one of the chaperones, in a concentration-dependent fashion (Fig. 1C). Notably, D2 activity half-life was not affected by 4-PBA (Fig. 1D;  $t_{1/2} \sim 47$  minutes), which suggests a transcriptional effect. In fact, treatment with TUDCA or 4-PBA increased Dio2 mRNA levels between 1.5 to 3 fold, respectively (Fig. 1E).

### 3.2 Chemical chaperones increase energy expenditure in primary cultures of brown adipocytes

Induction of D2-generated T3 accelerates the rate of energy expenditure in cell and animal models [15–18]. Here, we exposed primary murine brown adipocytes to 4-PBA or TUDCA for 24h, with D2 activity increasing 3–5-fold in response to either chemical chaperone (Fig. 2A). This induction was coincidental with acceleration in the rate of oxygen consumption that took place between 24–72h (Fig. 2B–C). Remarkably, this effect was greatly reduced (4-PBA; Fig. 2B) or abolished (TUDCA; Fig. 2C) when *Dio2*<sup>-/-</sup> brown adipocytes were used, indicating that D2 is a critical component in the chemical chaperone-induced acceleration in energy expenditure.

Next, we analyzed the expression of a number of metabolically relevant genes in brown adipocytes treated with the chemical chaperones. Exposure to either molecule increased the expression of *Ucp-1* and *Ucp-3* by about 3-fold, whereas only treatment with 4-PBA induced *Cpt-1*, catalase, *Glut-4* and *Pgc-1 $\alpha$*  mRNA levels (Fig. 2D). In the *Dio2*<sup>-/-</sup> brown adipocytes, the induction of these genes was markedly limited or abolished, with the exception of *Glut4* (Fig. 2D). Regardless of the cell type, both compounds were only active when used at  $\mu\text{M}$  concentrations (Fig. 1A, B and 2A), well within the range at which the metabolic effects of these molecules were reported [9]

### 3.3 D2 is critical for the metabolic improvement induced by chemical chaperones in mice

Next, we studied the metabolic profile of mice kept on a chow diet and treated with 0.5mg/kg BW TUDCA for 7 days. While treatment with TUDCA failed to promote changes in body weight, body composition and oxygen consumption (Table 3 and Fig. 3A–B), it significantly reduced the RQ of wild type mice by about 5% ( $p < 0.05$ ), indicating an increase in oxidation of fatty acids (Fig. 3D). This effect was lost in the TUDCA-treated *Dio2*<sup>-/-</sup> mice (Fig. 3D). Food intake was only minimally affected by treatment with TUDCA (Table 3). Accordingly, treatment with TUDCA was associated with doubling of D2 activity in BAT whereas in the pituitary gland D2 activity remained unchanged (Table 2). At the same time, no substantial changes were detected in the T4 or T3 serum concentrations of TUDCA-treated animals (Table 2). When placed on a high fat diet for 3 weeks, both wild type and *Dio2*<sup>-/-</sup> mice developed glucose intolerance (Fig. 4A and B). As previously reported [9], treatment with 0.5mg/kg BW TUDCA normalized glucose tolerance (Fig. 4A) but, remarkably, the same was not observed in the *Dio2*<sup>-/-</sup> animals (Fig. 4B).

## 4. DISCUSSION

Given the observation that the chemical chaperones TUDCA and 4-PBA stimulate D2 activity via *Dio2* mRNA accumulation (Fig. 1E) and affect key metabolic genes in a D2-dependent manner (Fig. 2D) we hypothesize that D2 is critical for the beneficial metabolic effects associated with chemical chaperones action. Our results support this hypothesis and identified D2 as a key point targetable by pharmacological intervention via the chemical chaperones. This is also supported by our previous studies [5,15,18], indicating that transcriptional induction of the *Dio2* gene plays a metabolic role. Here we expand this concept by providing a link between D2's activation by chemical chaperones and cell-specific thyroid hormone activation.

The expression of the *Dio2* gene is regulated by several factors, including NfκB [19] and cAMP [20,21], the latter being the main player mediating the 5-fold increase in the D2 gene seen in BAT during cold-induced adaptive thermogenesis [21]. D2 is also regulated post-transcriptionally by structural destabilization followed by ubiquitination [22–24]. Thus, it seemed logical to hypothesize that chemical chaperones could stabilize D2 protein and increase T3 production. However, both chemical chaperones increased D2 activity and intracellular T3 production (Fig 1) based on a *Dio2* transcriptional response rather than prolongation of D2 protein turnover rate (Fig 1D). It is not clear what the underlying mechanisms leading to increased *Dio2* mRNA levels are, but TUDCA has been shown to activate major signaling pathways including cAMP, PI3 kinase, p38 MAPK and ERK [18,25,26] whereas 4PBA is implicated in the activation of C-JNK [27] and PPARγ signaling pathways [28], as well as in the regulation of heat shock protein [29] and anti-microbial peptides (AMPs) [30].

By accelerating D2 expression one would expect increased T3 production. Accordingly, exposure of brown adipocytes to either 4-PBA or TUDCA accelerated oxygen consumption (Fig. 2B–C) and increased expression of metabolically relevant genes such as *Ucp-1*, *Ucp-3*, *Pgc-1α* (Fig. 2D), which are known to be T3 responsive [15,18]. This supports the concept that D2-expressing cells treated with chemical chaperones have increased thyroid hormone signaling. Remarkably, *Dio2*<sup>-/-</sup> brown adipocytes were much less sensitive to exposure to both chemical chaperones (Fig. 2B–D), where most T3 responsive genes had a lesser induction and also a limited acceleration of oxygen consumption (Fig. 2B). At the same time, the fact that exposure to 4-PBA or TUDCA increased *Glut4* expression in wild type and *Dio2*<sup>-/-</sup> brown adipocytes (Fig. 2D) indicates that this is not a T3-sensitive pathway and is likely to explain at least some of the metabolic effects of TUDCA in the *Dio2*<sup>-/-</sup> animals (Fig. 4A and B).

These cellular data set the stage for *in vivo* studies with TUDCA, which is suitable for *in vivo* administration [9,31]. D2 stimulation *in vivo* was associated with a reduction in the RQ (Fig. 3CD), indicating increased fatty acid oxidation. This happened without changes in serum T4 or T3 concentrations (Table 2), illustrating the high tissue-specificity of D2-mediated mechanisms. Such as observed previously [9], treatment with TUDCA improved systemic glucose homeostasis in animals placed on a high fat diet (Fig. 4A). Since these metabolic effects were minimized in the *Dio2*<sup>-/-</sup> mice (Fig. 4B), it is clear that D2 plays an important role in this process. Because the global *Dio2*<sup>-/-</sup> mouse was used in these experiments, it is harder to identify the specific D2-expressing tissues targeted by TUDCA that could be responsible for the metabolic phenotype. However, BAT is a likely candidate given that brown adipocytes and BAT respond to these chemical chaperones *in vitro* (Fig. 2; Table 2), a tissue known for the interplay between D2, PGC1 $\alpha$  and UCP-1 [4].

In the present studies we linked D2-mediated T3 production with acceleration of cellular oxygen consumption. In animals, induction of D2 was associated with increased fatty acid oxidation and improvement of glucose parameters. Given that BAT expresses high levels of D2 one would attribute most of the D2-mediated effects of TUDCA to stimulation of BAT [15,18]. However, other D2-expressing metabolically relevant tissues such as skeletal muscle could be involved as well [5]. BAT and glucose homeostasis seem to be particularly affected by thyroid hormone, probably due to its effect to increase insulin signaling [32].

## Acknowledgments

We are grateful to John W. Harney for the outstanding technical assistance. Grant support from NIDDK (DK65055). W.S.S. is a fellow of the Pew Charitable Trusts Foundation.

## Nonstandard Abbreviations Used

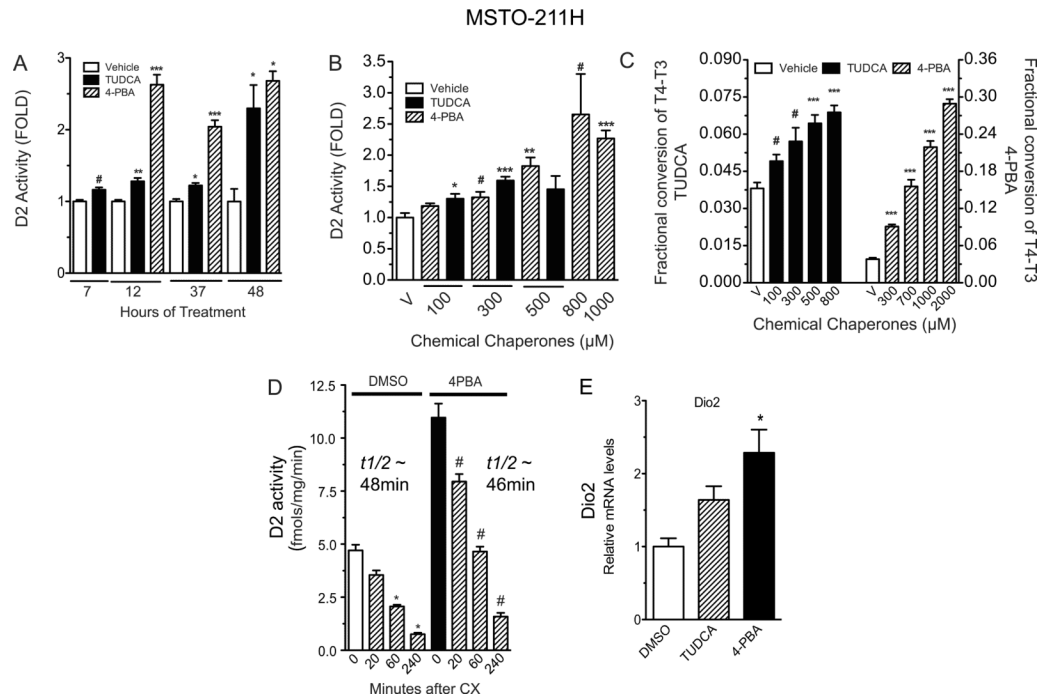
<b>D2</b>	type 2 deiodinase
<b>CPT-1</b>	carnitine palmitoyl transferase-1
<b>GLUT4</b>	glucose transporter 4
<b>UCP-1</b>	uncoupling protein 1
<b>UCP-3</b>	uncoupling protein 3
<b>Dio 2</b>	type 2 deiodinase gene
<b>mt TFA</b>	mitochondrial transcription factor 1
<b>PGC-1<math>\alpha</math></b>	peroxysome proliferator-activated receptor gamma coactivator 1 $\alpha$
<b>PGC-1<math>\beta</math></b>	peroxysome proliferator-activated receptor gamma coactivator 1 $\beta$
<b>TUDCA</b>	tauroursodeoxycholic acid
<b>4-PBA</b>	4-phenylbutyric acid
<b>%VO<sub>2</sub></b>	volume of oxygen
<b>RQ</b>	respiratory quotient

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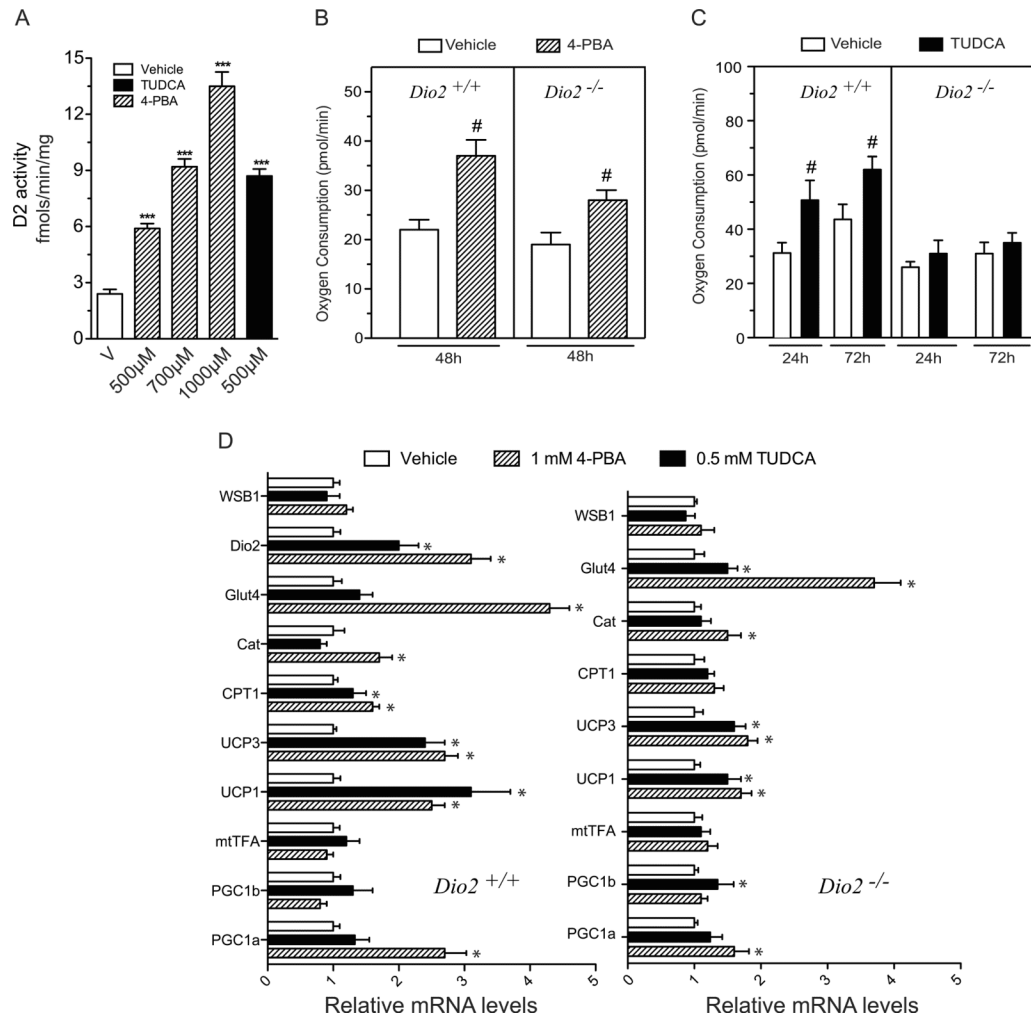
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**Figure 1. Tauroursodeoxycholic acid and 4-phenylbutyric acid stimulate D2 message, activity and T3 production in intact cells**

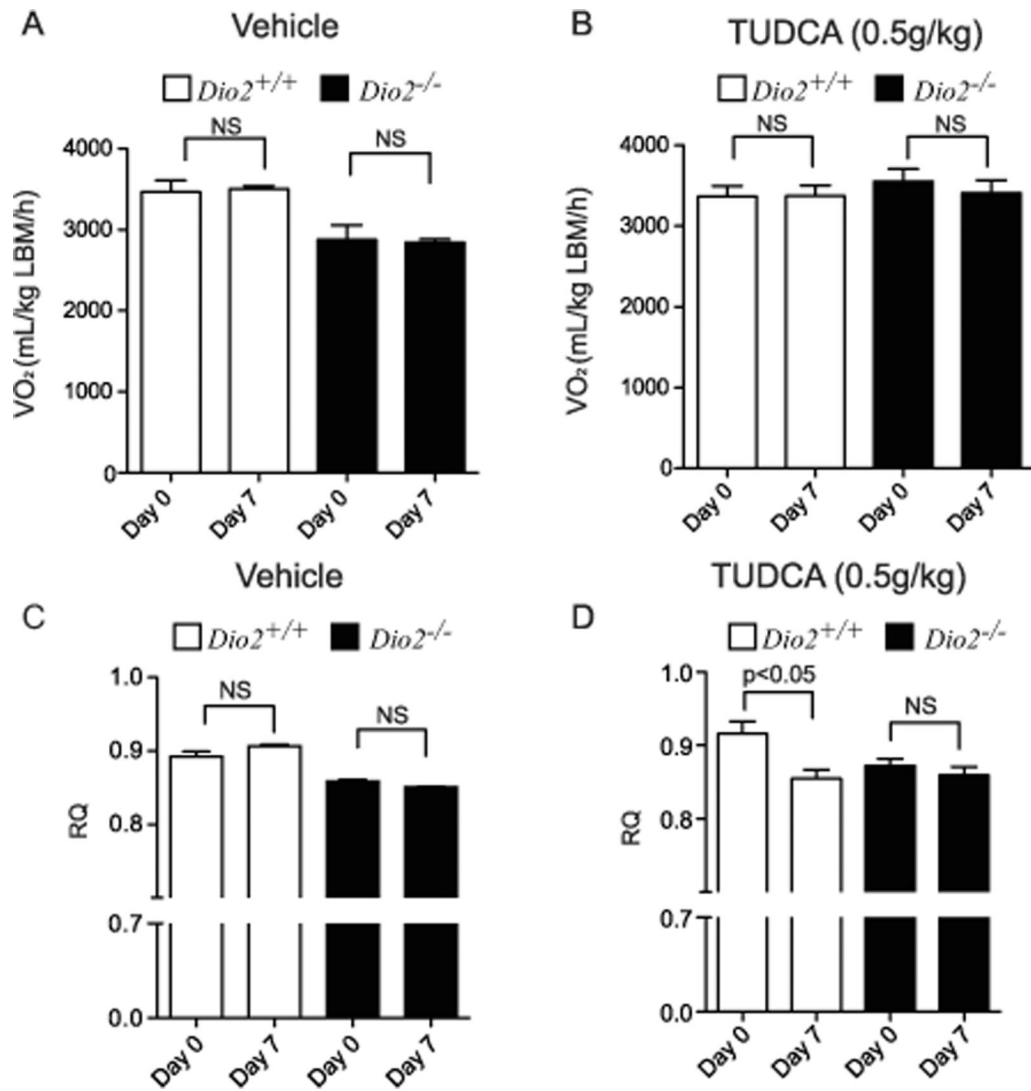
(A) D2 activity in MSTO-211H cells exposed to 500  $\mu$ M TUDCA or 1000  $\mu$ M 4-PBA for the indicated times. (B) Dose-response curve of D2 activity in MSTO-211H cells treated for 24h with the indicated amounts of 4-PBA or TUDCA. (C) T3 production in MSTO-211H cells treated for 48h with TUDCA or 4-PBA at indicated doses. (D) D2 activity in MSTO-211H cells treated with 4-PBA and/or cyclohexamide for the indicated times. (E) Dio2 gene expression in MSTO-211H cells treated for 24h with 500 $\mu$ M TUDCA or 1mM 4-PBA. Values are the mean  $\pm$  SEM of 3–6 experiments. # P<0.05, \* P<0.01, \*\* P<0.001, \*\*\* P<0.0001 vs. vehicle treated cells.





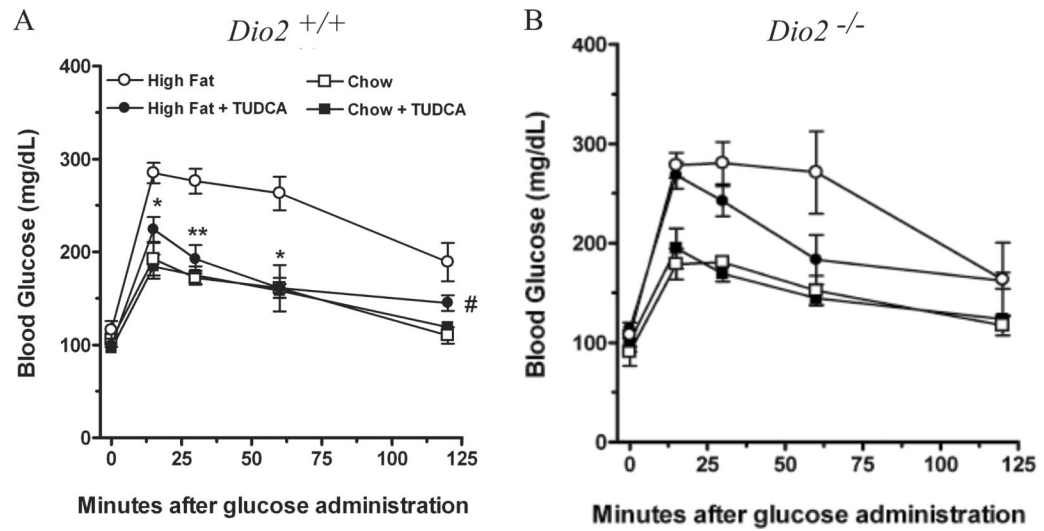
**Figure 2. TUDCA and 4-PBA increase energy expenditure and modify gene expression in brown adipocytes**

(A) D2 activity in differentiated wild type primary brown adipocytes exposed to increasing amounts of 4-PBA or 500 $\mu$ M TUDCA for 24h. (B) and (C), oxygen consumption in wild type or *Dio2*<sup>-/-</sup> primary brown adipocytes treated with 1mM 4-PBA for 48h or 500 $\mu$ M TUDCA for 24 or 72h. (D) Relative mRNA levels in TUDCA or 4-PBA-treated wild type or *Dio2*<sup>-/-</sup> primary brown adipocytes at indicated doses. Values are the mean  $\pm$  SEM of 6–10 samples of at least 2 experiments. # P<0.05, \* P<0.01 or \*\*\* P<0.0001 vs. vehicle-treated cells.



**Figure 3. Impact of tauroursodeoxycholic acid on indirect calorimetry of wild type or  $Dio2^{-/-}$  animals**

Oxygen consumption (A–B) and respiratory quotient (RQ) (C–D) in mice on chow diet before and after treatment with 0.5mg/g BW TUDCA for 7 days. For (A–D) all values were calculated as the area under the curve of measurements made on the first or last 24h of treatment, and are presented as the mean  $\pm$  SEM of 3 animals. In (D),  $P < 0.05$  vs.  $Dio2^{-/-}$  by two-tail Students' t-test.



**Figure 4. Tauroursodeoxycholic acid improves glucose tolerance in wild type but not in *Dio2*<sup>-/-</sup> mice**

Glucose tolerance test in WT (**A**) and *Dio2*<sup>-/-</sup> (**B**) placed on a chow or high fat diet. All values are the mean  $\pm$  SEM of 4 animals, where \*  $P < 0.01$ , \*\*  $P < 0.001$  and #  $P < 0.05$  vs. high fat diet group. These experiments were repeated at least 2 times.

**Table 1**

D1 activity in HepG2 cells exposed to 4-PBA or TUDCA

Treatment	D1 activity (pmols/min*mg protein)
Vehicle	2.9 ± 0.05
500µM TUDCA	2.7 ± 0.38
500µM 4-PBA	2.5 ± 0.21
1000µM 4-PBA	2.7 ± 0.05

Cells were treated for 24h with vehicle (DMSO) or the indicated concentrations of TUDCA or 4-PBA. Values are the mean ± SEM of 3 different samples. All values are not statistically different.

**Table 2**

Serum concentrations of T4 and T3, D2 activity in BAT and pituitary gland of mice treated with TUDCA

Treatment	D2 activity (fmols/min*mg protein)	T4 (ng/ml)	T3 (ng/ml)
BAT-vehicle	1.9 ± 0.12	-----	-----
BAT-TUDCA	4.3 ± 0.78*	-----	-----
Pit-vehicle	2.6 ± 0.73	-----	-----
Pit-TUDCA	2.9 ± 0.64	-----	-----
Serum-vehicle	-----	3.9 ± 0.61	0.56 ± 0.44
Serum-TUDCA	-----	4.3 ± 0.93	0.59 ± 0.69

Animals treated for 10 days with vehicle or TUDCA (0.5mg/g BW) and tissues were processed for D2 activity. Serum T4 and T3 were measured by RIA. Values are the mean ± SEM of 5 different animals.

\* p<0.05 vs. vehicle-treated animals.

**Table 3**

Body and food intake data of mice before and after treatment with TUDCA

Parameter	<i>Dio2<sup>+/+</sup></i>	<i>Dio2<sup>-/-</sup></i>
ΔBody weight gain (g)	1.57 ± 0.58	0.5 ± 0.53
ΔFood Intake (mg/g)	0.2 ± 0.59	-0.51 ± 0.14 <sup>*,**</sup>
Final body composition (% fat)	19.8 ± 1.9	17.9 ± 2.3

Animals were treated for 7 days with vehicle or TUDCA (0.5mg/g BW) and body weight and food intake were recorded daily and reported as the variation between before and after treatment. Body composition was measured by DEXA. Values are the mean ± SEM of 6 different animals.

\* p<0.05 vs. vehicle-treated animals by student's t test;

\*\* p<0.05 when paired student's t test is used to compare the same animal before and after treatment.