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# Thyroid hormone status regulates the expression of secretory phospholipases

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ABSTRACT

Thyroid hormone (T3) stimulates various metabolic pathways and the hepatic actions of T3 are mediated primarily through the thyroid hormone receptor beta (TRβ). Hypothyroidism has been linked with low grade inflammation, elevated risk of hepatic steatosis and atherosclerosis. Secretory phospholipases (sPLA2) are associated with inflammation, hyperlipidemia and atherosclerosis. Due to potential linkage between thyroid hormone and sPLA2, we investigated the effect of thyroid hormone status on the regulation of secretory phospholipases in mice, rats and human liver. T3 suppressed the expression of the sPLA2 group IIa (PLA2g2a) gene in the liver of BALB/c mice and C57BL/6 transgenic mice expressing the human PLA2g2a. PLA2g2a was elevated with hypothyroidism and high fat diets which may contribute to the low grade inflammation associated with hypothyroidism and diet induced obesity. We also examined the effects of the TRβ agonist eprotirome on hepatic gene regulation. We observed that eprotirome inhibited the expression of *selected sPLA2 genes* and furthermore the cytokine mediated induction PLA2g2a was suppressed. In addition, eprotirome induced genes involved in fatty acid oxidation and cholesterol clearance while inhibiting lipogenic genes. Our results indicate that in vivo thyroid hormone status regulates the abundance of sPLA2 and the inhibition of PLA2g2a by T3 is conserved across species. By regulating sPLA2 genes, T3 may impact processes associated with atherosclerosis and inflammation and TRβ agonists may ameliorate inflammation and hyperlipidemia.



## 1. Introduction

Thyroid hormone (T3) promotes reverse cholesterol transport, stimulates conversion of cholesterol to bile acids and enhances metabolic rate [1]. There are two isoforms of the thyroid hormone receptor (TR) including TRα and TRβ [2]. The TRα isoform is highly expressed in the heart and is associated with elevated cardiac function [3]. The lipid lowering actions of T3 are mediated primarily through the TRβ isoform which is predominant in liver [4].

Phospholipases A2 are a family of esterase enzymes which hydrolyze the second carbon of membrane phospholipids to release non-esterified free fatty acids and lysophospholipids [5]. The free fatty acid, arachidonic acid, is a precursor for inflammatory mediators such as eicosanoids [5]. PLA2 enzymes have been classified into three major groups: cytosolic phospholipase A2, secretory phospholipase A2 and calcium independent phospholipase A2. Phospholipase group IIa (PLA2g2a) is a secretory phospholipase that is highly expressed in the liver and macrophages [6]. Expression of the PLA2g2a gene is rapidly induced by cytokines, CCAAT enhancer binding protein β (C/EBPβ) and inflammatory signals [7]. High levels of PLA2g2a are associated with the progression of atherosclerosis and arthritis [8,9].

We found that T3 stimulates numerous metabolic genes in the liver including carnitine palmitoyltransferase 1a (CPT1a), phosphoenolpyruvate carboxykinase (PEPCK) and pyruvate dehydrogenase kinase 4 (PDK4) [10,11]. For these genes, the binding of the TRβ is essential for transcriptional activation. However, the stimulation of these genes also involves other transcription factors such as the CCAAT enhancer binding protein (C/EBPβ) as well as several coactivators including peroxisome proliferator activated receptor gamma coactivator (PGC-1α) and the deacetylase Sirtuin 1 (SIRT1) [12,13]. We recently reported that the rat PLA2g2a promoter has a negative thyroid hormone response element (nTRE) and the binding of liganded TRβ to this element recruits nuclear corepressors NCoR1 and SMRT to inhibit PLA2g2a gene transcription [14].

Over the past decade, a number of TRβ agonists have been developed that stimulate TRβ without impacting TRα [15]. These ligands are either selectively cleared by the liver or bind with greater affinity to TRβ. Therapeutically, the goal of these agonists is to enhance the lipid lowering actions of T3 without impacting the cardiovascular system. Several TRβ agonists such as sobetirome

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(GC-1) and eprotirome (KB2115) decreased circulating cholesterol without elevating heart rate. In a 2 week clinical trial, KB2115 lowered the LDL cholesterol by 40% [16]. In phase 2 clinical trials, eprotirome was administered to patients already on statin therapy. Following a 100 μg or 200 μg dose of KB2115 for 12 weeks, there was a 30% decrease in the LDL cholesterol and a significant reduction in triglycerides. There was no change in heart rate or muscle strength [17]. These studies highlight the potential value of TRβ agonists and the need for further study of their hepatic targets.

In present study, we examined effect of thyroid status and the ability of T3 and the TRβ selective agonist KB2115 to regulate sPLA2 the expression. Our data indicate that T3 negatively regulates *some* secretory phospholipases in mice, rats and human PLA2g2a transgenic mice models. The TRβ agonist KB2115 not only decreased the basal levels of sPLA2s but also the cytokine mediated induction of PLA2g2a. Genes involved in mitochondrial (CPT1a) and peroxisomal fatty acid oxidation (ABCD3, ACAA1) as well as in cholesterol metabolism like CYP7A1 and SRB-I were induced by KB2115. TRβ agonists may be useful in the treatment of low grade inflammation and fatty liver associated hepatic steatosis.

## 2. Materials and methods

### 2.1. Primary rat hepatocyte culture and treatment

Rat hepatocytes were prepared as described previously [11]. Hepatocytes were seeded in 60 mm plates at a density of $3 \times 10^6$ -cells/plate and maintained for 12 h in RPMI 1640 media with 10% fetal bovine serum. The next day, cells were washed twice with PBS and serum free RPMI 1640 media was added. The hepatocytes were treated with 100 nM $T_3$ or 1 μM of KB2115 for 24 h.

### 2.2. Animals and treatments

Hypothyroidism was induced in BALB/c mice and male Sprague Dawley rats by feeding an iodine-free diet containing 0.15% propylthiouracil (Teklad 95125) for 5 weeks. The rats and mice were given intraperitoneal injections of $T_3$ or KB2115 (0.33 mg/kg and 0.11 mg/kg of body weight respectively) and after 24 h a second dose of $T_3$/KB2115 was given. Animals were sacrificed after 24 h and livers were isolated for RNA and protein. For high fat diet experiments, BALB/c mice were divided into three groups: (1) chow fed (Teklad Diet 06101), (2) high fat diet (HFD) (TD 95217) and (3) hypothyroid (0.05% PTU) (TD 120714). The energy content of the HFD was 45% fat and 40% carbohydrate while the chow diet has 65% carbohydrate and 17% fat.

C57BL/6 IIA+ mice contained the human PLA2g2a gene under the regulation of its own promoter [9,18]. To induce hypothyroidism, PTU was added at 0.05% in the diet (TD 120714) for 6 weeks. Each group of mice contained seven female mice. C57BL/6 IIA+ were made hyperthyroid by administering two intraperitoneal injections of $T_3$ (0.11 mg/kg of body weight) for 2 days.

### 2.3. RNA extraction and real time PCR

RNA was extracted from rat liver and primary hepatocytes by RNA-STAT 60 (Tel-Test). The extracted RNA was further purified with the Qiagen RNeasy Mini Kit (74104). cDNA was made by reverse transcribed using Superscript III (Invitrogen). The parameters for RT-PCR were as follows: 95 °C for 5 min, 40 cycles of 95 °C for 15 s, 60 °C for 30 s and 72 °C for 10 s. 18S was used as a reference gene and the quantification of the PCR products was carried out using the ΔΔCt method. *The relative Ct values for the rat PLA2g2a were 25-27, mouse PLA2g2a 32-34 and human PLA2g2a 12–14*. The forward and reverse primers used for real time PCR are as follows: rat PLA2g2a FP catggcctttggctcaattcaggt, rat PLA2g2a RP acagtcat gagtcacacagcacca, rat PLA2g3 FP acagccttgaacttctggtccact, rat PLA2g3 RP gctttgagcaggttgaagcgttg, rat PLA2g5 FP aactgtgtggtcttg aacctccgt, rat PLA2g5 RP acacactctcatgcagcctaccat, and rat PLA2g1b qiagen (QT00179529) mouse PLA2g2a FP agcctcgatcatggcctttt, mouse PLA2g2a RP gccgaatcatttccccaaa, human PLA2g2a FP catggc ctttggctcaattcaggt, human PLA2g2a RP aggctggaaatctgctggatgtct.

### 2.4. ELISA

HepG2 cells were grown in 24 well plates ($0.2 \times 10^6$ cells/well) in DMEM media containing 10% fetal bovine serum (FBS). The following day, the cells were incubated in serum free DMEM media with 1 μM KB2115 or 10 ng/mL IL-6. After 24 h, cell culture media was centrifuged at 1200 rpm to remove cell debris. ELISA was performed for PLA2g2a using EIA Kit from Cayman Chemicals (585000).

### 2.5. Western blot

Proteins from rat livers were harvested by homogenizing the livers in RIPA buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 5 mM EDTA pH 8.0, 1% Triton, 1 mM benzamidine, 0.5 mM PMSF). An equal amount of protein was loaded on a 3–8% Tris-acetate acrylamide gel. Proteins were immunoblotted with primary antibodies PLA2g2a (Abcam) and Tubulin (Sigma) [14]. Immunoreactive proteins were detected using Super signal West femto chemiluminescent substrate (Thermo Scientific).

### 2.6. Transient transfections

HepG2 cells were transfected with 2 μg of −448/+58 rPLA2g2a luciferase reporter, 1 μg of an SV40-TRβ and 0.1 μg of TK-renilla by the calcium phosphate method. On the next day, the media was changed to serum free media and KB 2115 was added. Cells were lysed after 24 h and luciferase assays were conducted using the Promega Dual Luciferase. Luciferase values were normalized for renilla luciferase activity.

### 2.7. Lipid assessments

Plasma cholesterol and lipoproteins were measured in the University of Tennessee Endocrinology/Lipoprotein Laboratory.

### 2.8. Statistical analysis

All experiments were conducted 3–4 times and the error bar indicates SEM. The data was analyzed by Student's one/two tailed *t* test or ANOVA. *p* Values <0.05 were considered to be statistically significant.

## 3. Results

### 3.1. Thyroid hormone negatively regulates expression of secretory PLA2 in BALB/c and transgenic C57BL/6 IIA+ mice

Our first objective was to test whether the thyroid hormone status regulates the PLA2g2a gene in BALB/c mice. BALB/c mice were made hypothyroid by inclusion of 0.05% PTU in the diet. After 5 weeks, mice were given two injections of T3. Administration of T3 reduced the expression of the mouse PLA2g2a gene (Fig. 1A). Other genes such as PEPCK and PDK4 were induced by T3 (Fig. 1B and C), while CPT1a was not elevated by T3 in mice (Fig. 1D). The mouse CPT1a has a different pattern of tissue specific

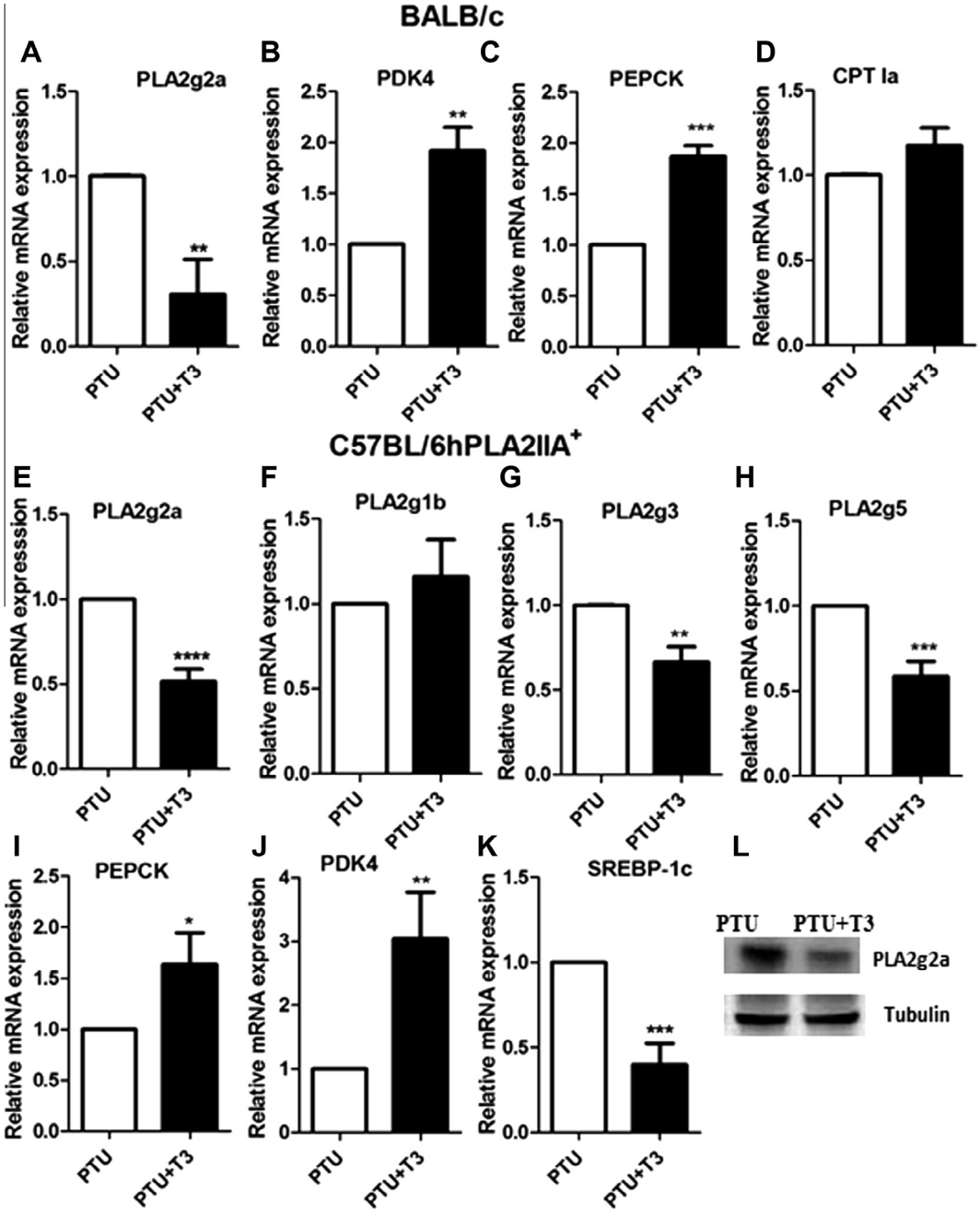


**Fig. 1.** Thyroid hormone status regulates expression of secretory PLA2 in BALB/c and C57BL/6 hPLA2 IIA+ mice. BALB/c mice were made hypothyroid or hyperthyroid as described in Section 2. Mice were sacrificed and liver RNA was harvested. The mRNA levels were measured for (A) PLA2g2a, (B) PDK4, (C) PEPCK and (D) CPT1a. Values are the average of RNA from 5 mice. C57BL/6 IIA+ mice were fed a PTU supplemented diet for 5 weeks. Mice were injected with T3 and RNA was harvested from the liver. The mRNA levels were measured for (E) PLA2g2a, (F) PLA2g1b, (G) PLA2g3, (H) PLA2g5, (I) PEPCK, (J) PDK4 and (K) SREBP-1c. (L) PLA2g2a protein abundance was measured by western blotting. Values are the average of RNA from 7 mice. The data are expressed as the mean of the fold induction by PTU + $T_3$ ± SEM of mRNA abundance relative to PTU treated mice (*$p$ value <0.05; **$p$ value <0.01; ***$p$ value <0.001; ****$p$ value <0.0001).

**Table 1**
Administration of T3 to hypothyroid C57BL/6 hPLA2gIIA+ mice alters lipid levels.

| Mice ($N$ = 7) | Cholesterol (mg/dL) | Triglycerides (mg/dL) | HDL (mg/dL) | LDL (mg/dL) | FT3 (pg/mL) |
|---|---|---|---|---|---|
| PTU Mice | 159 ± 7.1 | 55.2 ± 3.3 | 93.7 ± 2.8 | 54.2 ± 4.6 | 1.5 ± .30 |
| PTU + T3 Mice | 40.57 ± 4.3**** | 42.86 ± 4.5* | 31.29 ± 3.7**** | 1.5 ± 0.80**** | 18.5 ± 1.1**** |

C57BL/6 hPLA2gIIA+ mice were fed a PTU supplemented diet for 5 weeks. T3 was administered as described previously. Serum levels of free unbound $T_3$ (FT3), cholesterol, triglycerides, high density lipoproteins (HDL) and low density lipoproteins (LDL) were measured. The averages ± SEM are from seven PTU or seven T3 treated mice (**$p$ value <0.01).

* $p$ Value <0.05.

**** $p$ Value <0.0001.

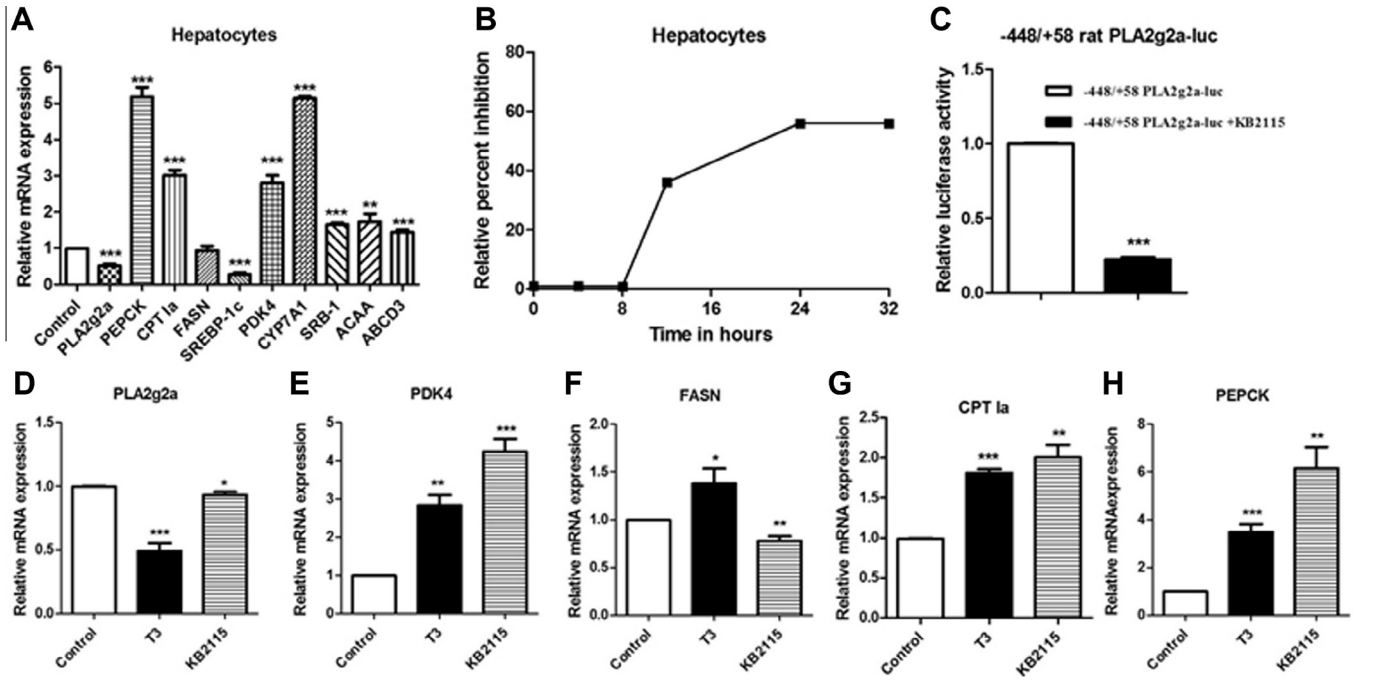


**Fig. 2.** Effect of the TRβ agonist KB2115 on hepatic gene regulation. (A) Rat hepatocytes were treated with 1 μM KB2115 for 24 h. RNA was assessed for mRNA abundance. (B) Rat hepatocytes were treated with KB2115 for various times and the relative inhibition of PLA2g2a expression was measured. (C) HepG2 cells were transfected with 2 μg of −448/+58 *rat* PLA2g2a luciferase, 1 μg of SV40-TRβ and 0.1 μg of TK-renilla and treated with or without KB2115 for 24 h. (D–H) HepG2 cells were treated with 100 nM $T_3$ or 1 μM KB2115 for 24 h. RNA was assessed for various genes. All experiments were repeated 4–6 times. The data are expressed as the mean of the fold induction ± SEM of mRNA abundance relative to treatment control cells (**p* value <0.05; ***p* value <0.01; ****p* value <0.001).

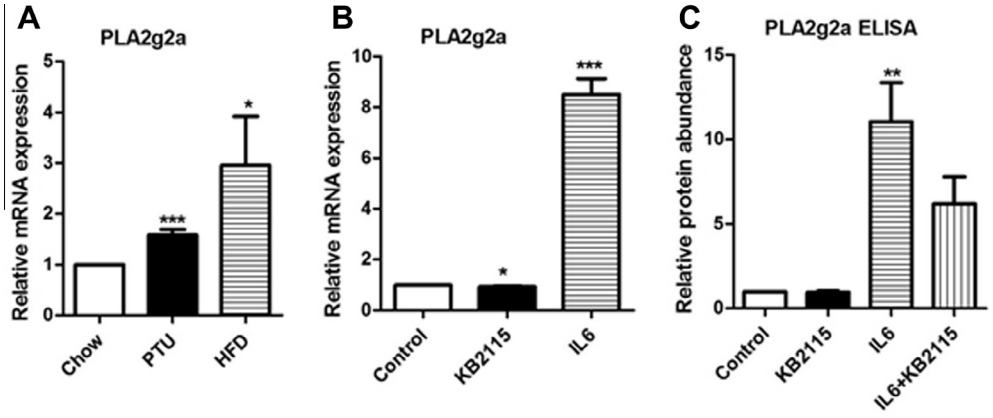


**Fig. 3.** Effect of hypothyroidism and high fat diets on PLA2g2a expression. (A) BALB/c mice were fed a chow diet, PTU diet or a high fat diet for 12 weeks. After 12 weeks, the mRNA levels of PLA2g2a were measured by RT-PCR. The data are expressed as the mean of the fold induction by hypothyroidism (PTU) or high fat diets (HFD) of PLA2g2a mRNA abundance relative to chow fed mice. (B) HepG2 cells were treated with 10 ng/mL IL-6 or 1 μM KB2115 or both for 24 h. PLA2g2a mRNA abundance was assessed. (C) Media was collected from HepG2 cells treated with IL-6 or KB2115 and the PLA2g2a levels were determined by ELISA. All experiments were repeated 3–4 times. Data are expressed as relative mRNA or protein abundance (**p* value <0.05; ***p* value <0.01; ****p* value <0.001).

expression than the rat or human genes so differential regulation of CPT1a gene by T3 was not unexpected.

To determine if T3 also regulates human PLA2g2a gene in vivo, we tested C57BL/6 transgenic C57BL/6 IIA+ mice expressing the human PLA2g2a gene under the regulation of the human promoter [9]. C57BL/6 mice do not express the PLA2g2a gene due to a frame shift mutation in exon 3 [19]. Following 5 weeks of the 0.05% PTU diet, mice were given two injections of T3. In the liver of the C57BL/6 IIA+ mice, T3 decreased the mRNA abundance of the PLA2g2a gene (Fig. 1E) demonstrating that the human PLA2g2a gene is inhibited by T3 in vivo. In addition, the PLA2g3 and PLA2g5 genes were negatively regulated by T3 (Fig. 1G and H), but PLA2g1b was not reduced (Fig. 1F). The PEPCK and PDK4 genes were elevated by T3 (Fig. 1I and J) while SREBP-1c was inhibited (Fig. 1K). We found that T3 reduced the PLA2g2a protein abundance in the transgenic mice (Fig. 1L). Overall, these results suggested that in vivo T3 negatively regulates the human and mouse PLA2g2a genes. Furthermore, we measured the effect of T3 on circulating plasma lipids. As shown in Table 1, administration of T3 strongly reduced the circulating levels of cholesterol, triglycerides, HDL and LDL.

### 3.2. Regulation of hepatic gene expression by TRβ agonist KB2115

Given the therapeutic advantages of TRβ agonists over T3, we investigated the effect of the TRβ agonist KB2115 on sPLA2 and other hepatic genes in rat hepatocytes and HepG2 cells. In hepato-

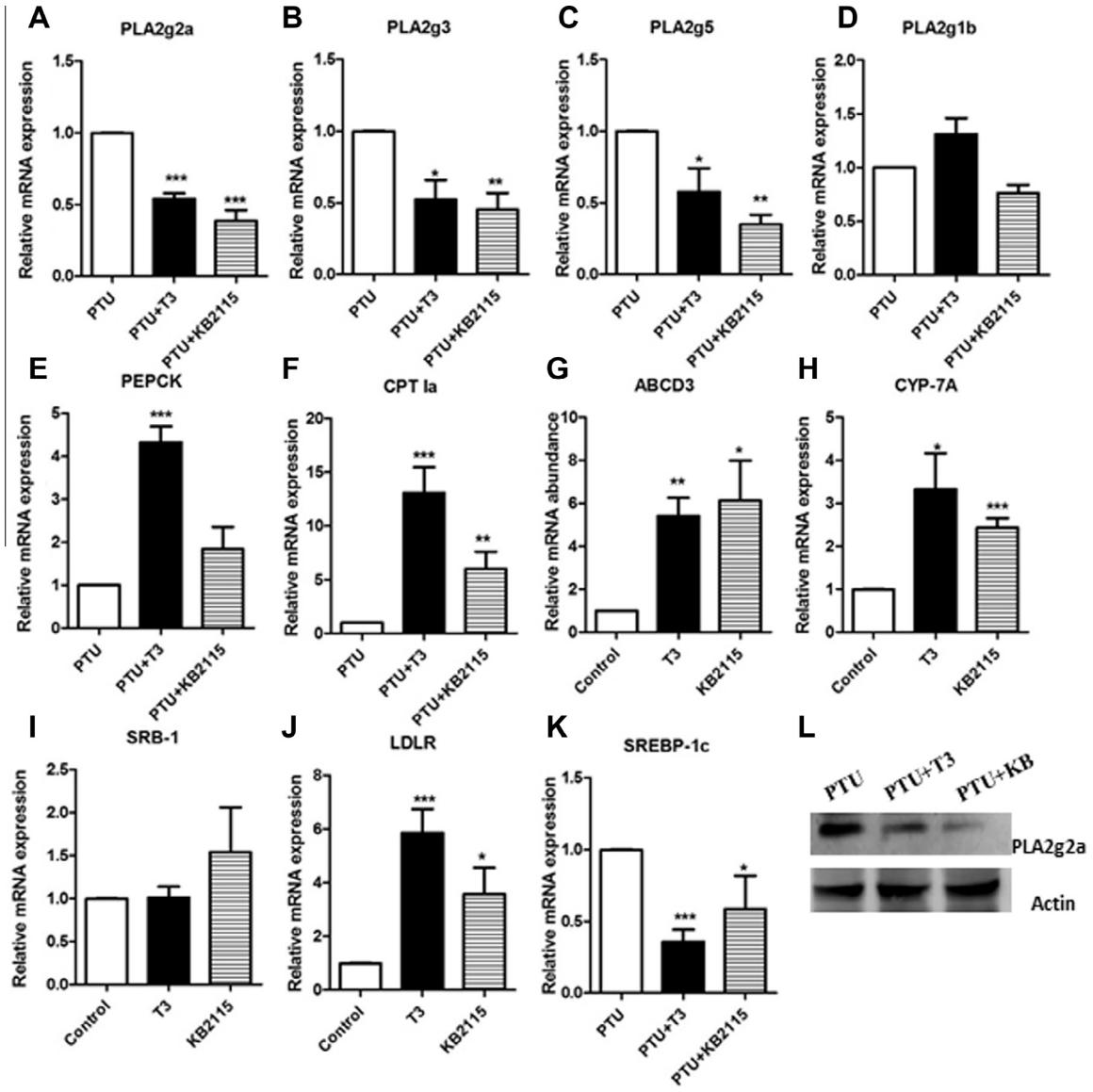


**Fig. 4.** Comparison of hepatic gene regulation by T3 and KB2115 in rats. Rats were made hypothyroid by adding 0.05% PTU in drinking water for 3 weeks. Hyperthyroidism was induced by i.p. injection of T3 (0.33 mg/kg body wt.) or KB2115 (0.33 mg/kg body wt.) for 2 days. The hepatic mRNA levels were measured for: (A) PLA2g2a, (B) PLA2g1b, (C) PLA2g3, (D) PLA2g5, (E) PEPCK, (F) CPT1a, (G) ABCD3, (H) CYP-7A, (I) SRB-I, (J) LDLR, and (K) SREBP-1c. (L) PLA2g2a protein abundance was measured by western. Values are the average of RNA from 5 mice. The data are expressed as the mean of the fold induction by PTU + $T_3$ or PTU + KB2115 ± SEM of mRNA abundance relative to PTU treated mice (*$p$ value <0.05; **$p$ value <0.01; ***$p$ value <0.001).

**Table 2**
Effect of T3 and KB2115 on the serum lipid levels.

| Rat ($N$ = 5) | Cholesterol (mg/dL) | Triglycerides (mg/dL) | HDL (mg/dL) | LDL (mg/dL) |
|---|---|---|---|---|
| PTU | 116 ± 3.8 | 29.4 ± 3.3 | 76.7 ± 2.8 | 34.2 ± 1.6 |
| PTU + T3 | 46.33 ± 4.6*** | 31.6 ± 3.3 | 28.5 ± 2.6*** | 11.3 ± 1.7*** |
| PTU + KB2115 | 84.2 ± 8.1** | 43.2.6 ± 3.9 | 48.7 ± 4.7*** | 27.2 ± 4.3 |

Rats were provided PTU supplemented water for 3 weeks. T3 and KB2115 were administered as described in the legend to Fig. 4. Serum levels of cholesterol, triglycerides, HDL and LDL were measured. The averages ± SEM are from seven PTU or seven T3 or KB2115 treated rats.
** $p$ Value <0.01.
*** $p$ Value <0.001.

cytes, KB2115 decreased PLA2g2a and inhibited the lipogenic gene SREBP-1c (Fig. 2A). The genes associated with cholesterol metabolism including the HDL receptor (SRB-I) and CYP7A1 which is involved in bile acid production were induced by KB2115. Genes involved in mitochondrial and peroxisomal fatty acid oxidation (CPT1a, ABCD3, and ACAA1) were stimulated as were the PEPCK and PDK4 genes (Fig. 2A). These results showed that the TRβ agonist regulated gene expression in a similar manner to T3. The time course of PLA2g2a inhibition by KB2115 is shown in Fig. 2B. To determine if KB2115 directly regulates PLA2g2a gene transcription, the −448/+58 *rat* PLA2g2a-luc promoter vector was transfected into HepG2 cells along with TRβ and cells were treated with

KB2115 for 24 h. KB2115 decreased the activity of −448/+58 PLA2g2a-luc indicating that PLA2g2a is inhibited at the promoter level (Fig. 2C). We compared the regulation of gene expression in HepG2 cells by T3 and KB2115 (Fig. 2D–G). The regulation of several genes was quite similar although KB2115 was not as effective as T3 in reducing the basal level of PLA2g2a gene (Fig. 2D).

### 3.3. Hypothyroidism, high fat diets and cytokines induces PLA2g2a gene

Our next experiments examined the effect of a high fat diet or hypothyroidism on the regulation of the PLA2g2a gene. We found that the PLA2g2a gene was induced 1.8-fold in hypothyroid mice (Fig. 3A). Since high fat diets induce low-grade inflammation, we tested whether abundance of PLA2g2a would be altered in mice fed a high fat diet. PLA2g2a was induced 3–4-fold (Fig. 3A). We next tested the effect of KB2115 on IL-6 mediated induction of the PLA2g2a gene. The PLA2g2a mRNA and protein levels were increased by interleukin 6 (IL-6) (Fig. 3B and C). KB2115 reduced the protein abundance of PLA2g2a following IL-6 treatment suggesting that KB2115 could decrease the cytokine mediated induction of PLA2g2a.

### 3.4. Comparison of hepatic gene regulation by T3 and KB2115 in rats

Finally, we evaluated and compared the in vivo regulation of secretory PLA2 genes and other hepatic genes by T3 and KB2115. The secretory PLA2 isoforms including PLA2g2a, PLA2g3, and PLA2g5 were significantly inhibited by KB2115 as well as T3. However, PLA2g1b was not inhibited by either T3 or KB2115 (Fig. 4A–D). *We also measured PLA2g10 but it was not significantly expressed in the liver of rats or mice (data not shown).* Genes involved in fatty acid oxidation were induced by KB2115 (Fig. 4F–G). CYP7A1, SRB-I and the LDL receptor (LDLR) were increased, while the lipogenic gene SREBP-1c was significantly inhibited (Fig. 4H–K). The protein levels of PLA2g2a were also inhibited by both T3 and KB2115 (Fig. 4L). Both T3 and KB2115 reduced the cholesterol, HDL and LDL levels in the rats although the effects of KB2115 were not as strong as T3 (Table 2). Neither T3 nor KB2115 altered the serum triglycerides. The actions of KB2115 are similar but not identical to T3.

## 4. Discussion

Hypothyroidism leads to hyperlipidemia and elevated cholesterol in patients [20]. Elevated PLA2g2a is associated with inflammation, hyperlipidemia and cardiovascular disease [21]. PLA2g2a promotes the movement of cholesterol from LDL into arterial plaques and has been associated with the progression of atherosclerosis. In these studies, we examined the T3 regulation of sPLA2 expression in three species. Our data indicate that the PLA2g2a gene is inhibited by T3 in rats, mice and humans. In both rats and mice, other secretory PLA2 isoforms including PLA2g3 and PLA2g5 were suppressed by T3.

Secretory PLA2 enzymes including PLA2g2a, PLA2g3 and PLA2g5 are pro-atherogenic due to their ability to oxidize LDL particles and promote the formation of lipid-laden foam cells from macrophages [6]. Introduction of PLA2g5 bone marrow cells into Ldlr-/-mice increased formation of atherosclerotic lesions. [22]. Likewise, PLA2g3 has been found to enhance the development of atherosclerosis and small LDL particles [23].

T3 has been studied primarily for its effects on metabolism and development. By regulating the expression of sPLA2 genes, T3 may impact genes and processes associated with atherosclerosis and inflammation. It is not known if T3 will inhibit secretory PLA2 genes in non-hepatic tissues, but the ability of T3 to decrease these sPLA2 isoforms suggests a potential avenue for slowing atherogenesis.

There has been considerable interest in the identification of TRβ selective agonists which have beneficial lipid lowering properties without cardiovascular consequences [1]. In primates, the TRβ agonist, GC-1, lowered cholesterol levels and reduced hyperlipidemia [1,17]. A related TRβ agonist, MB07811, induced CPT1a while inhibiting SREBP-1c [24]. MB07811 reversed the hepatic steatosis associated with high fat diets [25]. A recent study showed that GC-1 and KB2115 reduced hepatic steatosis in fat fed rats [26]. In terms of hepatic steatosis, part of the actions of TRβ agonist may include induction of CPT1a. In a limited human trial, administration of KB2115 for 12 weeks reduced cholesterol levels and improved the lipid profile [17]. In present study we found that KB2115 inhibited sPLA2 and the lipogenic gene SREBP-1c. Mitochondrial and peroxisomal fatty acid oxidation genes including CPT1a and ABCD3 were stimulated and similarly CYP7A1 involved in cholesterol metabolism was induced. These actions of KB2115 suggest that TRβ agonists may be useful in the low grade inflammation and hyperlipidemia associated with metabolic diseases and insulin resistance. TRβ ligands could improve the metabolic profile first by decreasing sPLA2 expression and the associated inflammatory response and secondly by reducing lipotoxicity via the stimulation of hepatic fatty acid oxidation and inhibition of lipogenesis. In addition to the effects on lipid and cholesterol metabolism, we postulate that the inhibition of sPLA2 gene by T3 may be another novel beneficial action of TRβ agonists.

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