RESEARCH

Multiple effects of cold exposure on livers of male mice

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Abstract

Cold exposure of mice is a common method to stimulate brown adipose tissue (BAT) activity and induce browning of white adipose tissue (WAT) that has beneficial effects on whole-body lipid metabolism, including reduced plasma triglyceride (TG) concentrations. The liver is a key regulatory organ in lipid metabolism as it can take up as well as oxidize fatty acids. The liver can also synthesize, store and secrete TGs in VLDL particles. The effects of cold exposure on murine hepatic lipid metabolism have not been addressed. Here, we report the effects of 24-h exposure to 4°C on parameters of hepatic lipid metabolism of male C57BL/6J mice. Cold exposure increased hepatic TG concentrations by 2-fold ($P<0.05$) but reduced hepatic lipogenic gene expression. Hepatic expression of genes encoding proteins involved in cholesterol synthesis and uptake such as the LDL receptor (LDLR) was significantly increased upon cold exposure. Hepatic expression of *Cyp7a1* encoding the rate-limiting enzyme in the classical bile acid (BA) synthesis pathway was increased by 4.3-fold ($P<0.05$). Hepatic BA concentrations and fecal BA excretion were increased by 2.8- and 1.3-fold, respectively ($P<0.05$ for both). VLDL-TG secretion was reduced by approximately 50% after 24 h of cold exposure ($P<0.05$). In conclusion, cold exposure has various, likely intertwined effects on the liver that should be taken into account when studying the effects of cold exposure on whole-body metabolism.

Key Words

- apolipoprotein
- cholesterol
- lipid
- liver

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Introduction

Two different types of adipose tissues are found in mammals: white adipose tissue (WAT) and brown adipose tissue (BAT). BAT plays an important role in thermogenesis by oxidizing fatty acids in order to generate heat instead of ATP molecules. A crucial player in thermogenesis is uncoupling protein-1 (UCP1), which uncouples ATP synthesis from oxidative phosphorylation. Instead, it redirects protons generated in the latter process directly back across the inner mitochondrial membrane, generating heat (Cannon & Nedergaard 2004, Kajimura *et al.* 2015). Recently it has become evident that certain WAT depots can gain BAT-like characteristics due to the appearance of brown adipocytes (Wu *et al.* 2012). Activation of BAT and 'browning' of WAT are both considered to increase

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whole-body energy expenditure, possibly leading to a reduction in body weight, which in turn may have a beneficial effect on obesity-related metabolic disorders such as diabetes mellitus type 2. The most relevant physiological way to activate BAT and induce 'browning' of WAT is by exposing animals to cold, resulting in an enhanced activity of the sympathetic nerves innervating BAT and WAT, thereby enhancing expression of proteins involved in thermogenesis and stimulating lipolysis (Himms-Hagen 1972).

Cold exposure in rodents has been shown to have profound effects on whole-body lipid metabolism. In particular, cold exposure lowers plasma triglyceride (TG) concentrations by enhancing lipolytic clearance of VLDL TG by BAT (Bartelt *et al.* 2011, Khedoe *et al.* 2015). Recently, it has been shown that cold exposure also affects whole-body cholesterol metabolism, for instance by inducing the reverse cholesterol transport by HDL particles (Bartelt *et al.* 2017) and by increasing the conversion of cholesterol to bile acids (BAs) (Worthmann *et al.* 2017).

Of interest, the liver is the main metabolic organ that controls whole-body TG, cholesterol and BA metabolism. With respect to TG homeostasis, the liver is able to take up fatty acids, oxidize fatty acids, synthesize fatty acids and TG *de novo*, and secrete TG in VLDL particles. Disturbances in the balance between these four pathways can result in an increased hepatic TG concentration (Willebrords *et al.* 2015). The liver is also crucial in maintaining cholesterol homeostasis since it can take up cholesterol from lipoprotein (remnants) by receptors such as the receptor (LDLR). In addition, the liver can synthesize cholesterol, convert cholesterol into BAs and excrete cholesterol via bile and in VLDL particles.

Despite the evident crucial role of the liver in controlling lipid metabolism, the effect of cold exposure on hepatic lipid metabolism has sparsely been addressed. Here, we report the effects of exposure to 4°C on various aspects of hepatic lipid metabolism in male mice.

Materials and methods

Animals

For the short-term cold exposure experiments, male C57Bl/6J mice were obtained from Charles River Laboratories at the age of 8 weeks and were acclimatized for 1 week under standard housing conditions before entering an experimental set-up. For the long-term cold exposure experiments, purebred 3- to 4-month old male C57Bl/6J mice were used that were obtained by in-house breeding. All animal experiments were performed with the approval of the Animal Ethics Committees of the Erasmus MC, Rotterdam, the Netherlands, or the Wageningen University, the Netherlands.

Cold exposure experiments

For the short-term cold exposure experiments, mice were individually housed in a temperature-controlled climate chamber (Bronson, Nieuwkuijk, the Netherlands) with a normal light/dark cycle at 23°C or 4°C for 24 h. At the end of this period, mice were terminated by cardiac puncture under isoflurane anesthesia between 13:00 h and 15:00 h and blood and various tissues were collected and snap-frozen in liquid nitrogen or fixed in 4% paraformaldehyde. Feces was collected during the 24-h period.

For the long-term cold exposure experiment, mice were individually housed at 28°C (thermoneutral temperature) or 4°C with a normal light/dark cycle for a period of 10 days. At the end of this period, mice were terminated with isoflurane and blood was collected. Following cervical dislocation, several tissues were collected and snap-frozen in liquid nitrogen. In a separate set of mice, blood was drawn via the tail vein at different days during the 10-day cold exposure for plasma TG measurements.

Treatment with CL316,243

To acutely activate BAT other than by cold exposure, mice were injected intraperitoneally with 1 mg/kg of the β3-adrenoreceptor agonist CL316,243 (Tocris Bioscience, Bristol, UK) dissolved in PBS or with PBS alone at 13:00 h, 17:00 h and 9:00 h. Five hours after the last injection, the mice were terminated by cardiac puncture under isoflurane anesthesia and blood and various tissues were collected.

VLDL-TG secretion experiments

To study VLDL-TG secretion, mice were deprived of food for the last 5 h in the climate chamber (i.e., from 09:00 h to 14:00 h) after which they were lightly sedated with isoflurane and an orbital blood sample was taken. Next, the mice received an orbital injection of 100 µL with 12.5% of the lipoprotein lipase inhibitor triton WR-1339 (Sigma-Aldrich) dissolved in PBS. Orbital blood samples were taken under light isoflurane sedation 30, 60, 120

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and 180 min after the triton WR-1339 injection. Mice were returned to the temperature-controlled climate chamber (23°C or 4°C) in between blood draws. The mice were terminated by cardiac puncture under isoflurane anesthesia directly after the last blood draw.

Plasma and hepatic lipid analysis and plasma T3 analysis

Plasma TG, cholesterol, alanine transaminase (ALT) and aspartate transaminase (AST) concentrations were measured with commercial available kits (ABX Pentra, Horiba, Irvine, CA). Hepatic concentrations of TGs and cholesterol were measured using commercial available kits (ABX Pentra) after lipid extraction according to Bligh and Dyer (Bligh & Dyer 1959). Plasma 3,3′,5-triiodothyronine (T3) concentrations were determined by radioimmunoassay as previously described (Friedrichsen *et al.* 2003).

Plasma and hepatic catecholamines

Livers and BAT were homogenized on ice in a glutathione buffer (60 mg/mL L-glutathione (Sigma-Aldrich) and 250 IU/mL heparin (LEO Pharma, Ballerup, Denmark) in water). Plasma was diluted 1:1 in glutathione buffer. Catecholamine extraction was performed according to the method followed by van der Hoorn *et al.* (van der Hoorn *et al.* 1989) with some modifications. To measure both norepinephrine and epinephrine, 50–200 µL liver homogenate was used. To control for equal recovery, alpha-methyl-norepinephrine (Sigma-Aldrich) was added as an internal standard to all samples. After extraction and derivatization with the fluorogenic agent 1,2-diphenylethylenediamine (Sigma-Aldrich), the samples were analyzed by HPLC with fluorometric detection (Shimadzu, 's Hertogenbosch, the Netherlands).

Hepatic glycogen and glucose-6-phosphate analysis

Hepatic glycogen concentrations were determined as described earlier (Feillet *et al.* 2016). In short, ~25 mg of liver was lysed in 0.5 M KOH at 95°C after which 25 µL 6% Na_2SO_4 and 750 µL methanol were added. Next, samples were split into 2 aliquots and glycogen was precipitated overnight at −80°C followed by centrifugation at 550***g*** for 30 min. Glycogen was either suspended in 200 µL 2 mg/mL amyloglucosidase (Sigma-Aldrich) in 50 mM sodium acetate (pH 4.8) or in 200 µL 50 mM sodium acetate (pH 4.8), incubated for 1 h at 37°C and centrifuged at 21,000***g*** for 1 min. The glucose concentration of 5 µL 2-fold diluted supernatant was measured using a glucose assay kit (Glucose LiquiColor, InstruChemie, Delfzijl, the Netherlands) according to the manufacturer's protocol. Glucose coming from glycogen was calculated as (total glucose) – (free glucose) for each sample.

Hepatic glucose-6-phosphate (G6P) concentrations were determined in ~50 mg liver tissue. Tissue samples were lysed by sonication in 750 µL ice-cold 5% $HClO_4$ after which proteins were precipitated by centrifugation at 18,000***g*** for 5 min. To 400 µL supernatant, 150 µL 0.3 M MOPS in 2 M KOH was added, followed by centrifugation at 18,000***g*** for 5 min. Of the supernatant, 125 µL was mixed with 125 µL 0.4 M triethanolamine buffer, pH 7.6 enriched with 400 µM NADP+ (Sigma-Aldrich) and 20 µM MgCl in a white 96-well plate, and the fluorescent signal was determined at 255 nm excitation, 460 nm emission. Ten minutes after the addition of 3 µL 20 U/mL *Leuconostoc mesenteroides* glucose-6-phosphate dehydrogenase (Sigma-Aldrich), the fluorescent signal was determined again. Next, 2 µL 1 mM D-glucose 6-phosphate disodium salt hydrate (Sigma-Aldrich) was added and the fluorescent signal was determined 10 min later. The increase in fluorescence upon the last addition was used to calculate the amount of G6P in the tissues.

Plasma and hepatic bile acids

Plasma BAs were measured using a colorimetric assay kit (Diazyme Laboratories, Poway, CA). For hepatic BA measurements, 120 µL of a 15% liver homogenate in saline was added to 140 µL methanol and 12 µL 10 M NaOH. This mixture was heated at 80°C for 2 h, cooled to room temperature, combined with 1 mL water and centrifuged at 670***g*** for 10 min. The BAs in the supernatant were determined using the colorimetric assay kit.

Fecal bile acids

Feces was air-dried, weighed, crushed and homogenized. To determine the fecal BA concentration, 100 mg pulverized feces was added to 2 mL 0.1 M NaOH and incubated at 60°C for 1 h. After cooling, 4 mL water was added and the mixture was homogenized and centrifuged at 20,000 g for 20 min. The supernatant was loaded onto a reverse-phase 30 mg SPE cartridge (Chromabond C18 cartridges, Macherey-Nagel, Düren, Germany; 30 µm bed size), pre-conditioned with consecutively 5 mL methanol and 5 mL

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water. Next, the cartridge was rinsed consecutively with 20 mL water, 10 mL hexane and again 20 mL water. Next, BAs were eluted with 5 mL methanol and this fraction was dried at 50°C under N_2. The samples were dissolved in 150 µL methanol and the BA concentration measured using the colorimetric assay kit.

VLDL isolation and determination of lipid composition

Nascent VLDL particles were isolated from plasma obtained by cardiac puncture after the VLDL secretion experiment. For this, 300 µL plasma was added to 3700 µL PBS and centrifuged at 160,000***g*** for 17 h, and the top layer containing the VLDL fraction was collected. The concentrations of TG, total cholesterol, free cholesterol and phospholipids in the isolated VLDL fractions were measured using commercial available kits (ABX Pentra for TG, DiaSys for cholesterol and phospholipids). The VLDL size was calculated using the formula of Fraser (Fraser 1970) modified by Harris *et al.* (Harris *et al.* 1997): Diameter(nm) = 60 × ((0.211 × TG/PL) + 0.27). In this formula, TG is the relative TG concentration and PL the relative phospholipid concentration.

Volumes of VLDL containing equal amounts of TG were extracted with methanol and cold ether to remove lipids. The remaining VLDL proteins were dissolved in Laemmli sample buffer (Bio-Rad Laboratories) supplemented with 100 mM Dithiothreitol (Sigma-Aldrich), boiled for 10 min, subjected to electrophoresis on 4.5% SDS gels and blotted onto a nitrocellulose membrane. Membranes were blocked in PBS containing 3% nonfat powdered skim milk before an overnight incubation at 4°C with an antibody against apolipoprotein B raised in rabbit (NB200-527; Novus Biologicals, Abingdon, Oxon, UK) in PBS containing 0.1% tween 20 and 3% nonfat powdered skim milk. Next, membranes were washed and incubated for 1 h at room temperature with a goat-anti-rabbit IRDye 800 secondary antibody (LI-COR, Leusden, The Netherlands) in PBS containing 0.1% tween 20 and 3% nonfat powdered skim milk.

Gene expression analysis

Total RNA isolation from mouse tissues and subsequent DNAse treatment and reverse transcription was performed as described previously (van den Beukel *et al.* 2014). Gene expression was measured using quantitative RT-PCR with SYBRgreen master mix (Applied Biosystems) and an ABI Prism 7900 Sequence Detection System (Applied Biosystems). The housekeeping genes 18S ribosomal RNA (*Rn18s*) and β2-microglobulin (*B2m*) for BAT and WAT or *Rn18s* and β-actin (*Actb*) for liver were used to normalize expression levels using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen 2001). Expression of the housekeeping genes was not influenced by housing temperature. Primer sequences used for all target genes are listed in Table 1.

Histology and immunohistochemistry

For hematoxylin and eosin staining, 4 and 8 µm sections of liver and BAT fixed in paraformaldehyde and embedded in paraffin were used. Sections were mounted on microscope slides (Thermo Scientific) and kept overnight at 37°C, deparaffinized in xylene and subsequently stained. For immunohistochemistry, sections were mounted on superfrost plus microscope slides (Thermo Scientific), kept at 37°C for at least 10 h before staining for extra adherence. Sections were kept at 60°C for 1 h and subsequently deparaffinized in xylene for 6 min, rinsed twice in 100% EtOH, put in methanol containing 3% H_2O_2 to block endogenous peroxidase activity and rinsed in demineralized water. Heat-induced antigen retrieval in NaOH buffered citric acid (pH 6.0) was applied. Sections were blocked with 5% normal goat serum (Dako) in PBS for 5 min, rinsed in PBS and then incubated overnight with either an antibody directed against glutamine synthase (GS; 1:100; BD Biosciences, Breda, the Netherlands) or an antibody against perilipin-2 (Pln2; 1:200; Novus Biologicals) at 4°C. BrightVision-poly-HRP-anti mouse/rabbit/rat IgG (Immunologic, Duiven, the Netherlands) in a 1:2 dilution in PBS was added for 30 min at room temperature as secondary antibody and peroxidase activity was developed with 0.07% 3,3-diaminobenzidine-tetrahydrochloride (Sigma-Aldrich) with subsequent counterstaining with hematoxylin.

Statistical analysis

Statistics were performed with GraphPad Prism (Version 5, GraphPad Software, Inc.). The two groups of mice were compared using an unpaired *t*-test. $P<0.05$ was considered statistically significant. For the plasma TG concentrations during the 10 days of exposure to 4°C, a one-way ANOVA was performed and differences between all time points were studied with Tukey's post hoc test in which $P<0.05$ was considered significant.

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Table 1 Sequences of primers.

Gene	Accession no.	Forward primer	Reverse primer
Abcg5	NM_031884	TGGCCCTGCTCAGCATCT	ATTTTTAAAGGAATGGGCATCTCTT
Abcg8	XM_006524826	AAGACGGGCTGTACACTGCT	AGTAGATGGGCATCGCGTAG
Acaca	XM_006531958	GGATGTGGATGATGGTCTGA	AGGCCTTGATCATCACTGGA
Actb	NM_007393	AAGGCCAACCGTGAAAAGAT	GTGGTACGACCAGAGGCATAC
Apob	NM_009693	AAACATGCAGAGCTACTTTGGAG	TTTAGGATCACTTCCTGGTCAAA
B2m	NM_009735	ATCCAAATGCTGAAGAACGG	CAGTCTCAGTGGGGGTGAAT
Chrebp	NM_021455	CACTCAGGGAATACACGCCTAC	ATCTTGGTCTTAGGGTCTTCAGG
Chrebpb	JQ437838	TCTGCAGATCGCGTGGAG	CTTGTCCCGGCATAGCAAC
Cpt1a	NM_009948	TGCCTTTACATCGTCTCCAA	GGCTCCAGGGTTCAGAAAGT
Cyp7a1	NM_007824	CTGTCATACCACAAAGTCTTATGTCA	ATGCTTCTGTGTCCAAATGCC
Cyp7b1	NM_007825	CCTCTTTCCTCCACTCATACACAA	GAAGCGATCGAACCTAAATTCCTT
Cyp8b1	NM_010012	TCCTCAGGGTGGTACAGGAG	GATAGGGGAAGAGAGCCACC
Cyp27a1	NM_024264	TACACCAATGTGAATCTGGC	TAACCTCGTTTAAGGCATCC
Elovl3	NM_007703	TCCGCGTTCTCATGTAGGTCT	GGACCTGATGCAACCCTATGA
Elovl6	NM_130450	CAGCAAAGCACCCGAACTA	AGGAGCACAGTGATGTGGTG
Fasn	NM_007988	GCTGCTGTTGGAAGTCAGC	AGTGTTCGTTCCTCGGAGTG
Fgf21	NM_020013	CTGGGGGTCTACCAAGCATA	CACCCAGGATTTGAATGACC
Gpat1	NM_008149	AGCAAGTCCTGCGCTATCAT	CTCGTGTGGGTGATTGTGAC
Hmgcr	NM_008255	CCGGCAACAACAAGATCTGTG	ATGTACAGGATGGCGATGCA
Ldlr	NM_010700	GCATCAGCTTGGACAAGGTGT	GGGAACAGCCACCATTGTTG
Lpin2	NM_001164885	CTGCTTATCTTGCCACCTC	CTGCTTATCTTGCCACCTC
Mcad	NM_007382	GATCGCAATGGGTGCTTTTGATAGAA	AGCTGATTGGCAATGTCTCCAGCAAA
Mttp	NM_008642	CGAGTGAAAAATCGGGTGGC	GGCTTCAGCCTTGTCCATCT
Nrg4	NM_032002	CCCAGCCCATTCTGTAGGTG	ACCACGAAAGCTGCCGACAG
Pcsk9	NM_153565	CACCATCACCGACTTCAACA	GTCACACTTGCTCGCCTGT
Pklr	NM_013631	GGGGTGACCTTGGCATTGAG	TTACAGCCTCCACGGGGAAA
Plin5	NM_001077348	TCCTGCCCGTCAAAGGGATCTGA	GGACATTCTGCTGTGTGGCGCT
Ppara	NM_011144	GCCTTCCCTGTGAACTGACG	AGAGCGCTAAGCTGTGATGA
Ppargc1a	NM_008904	CCCTGCCATTGTTAAGACC	TGCTGCTGTTCCTGTTTTC
Rn18s	NR_003278	GTAACCCGTTGAACCCCATT	CCATCCAATCGGTAGTAGCG
Scd1	NM_009127	AGATCTCCAGTTCTTACACGACCAC	GACGGATGTCTTCTTCCAGGTG
Srebp1c	XM_006532716	GGAGCCATGGATTGCACATT	CCTGTCTCACCCCCAGCATA
Srebp2	NM_033218	CCAAAGAAGGAGAGAGGCGG	CGCCAGACTTGTGCATCTTG
St3gal5	NM_001035228	GGTGTTGAGGTGGGAGGAGAG	GATGGACTAGCAGAAAGGGGTTATGAA
Thrsp	NM_009381	ATGCAAGTGCTAACGAAACGC	CCTGCCATTCCTCCCTTGG
Ucp1	NM_009463	GGCCTCTACGACTCAGTCCA	TAAGCCGGCTGAGATCTTGT

Results

Cold exposure activates BAT and stimulates browning of inguinal WAT

Exposure to 4°C is a common method to activate BAT and stimulate browning of WAT depots in mouse models. Exposure of male C57Bl/6J mice to 4°C for 24 h did not affect the weight of the interscapular BAT and inguinal and gonadal WAT depots, but resulted in a significant reduction in body weight, despite a 1.54±0.09 fold increase in food intake (Table 2). As expected, 24-h exposure to 4°C significantly induced interscapular BAT mRNA expression of peroxisome proliferator-activated receptor-gamma co-activator 1a (*Ppargc1a*) and *Ucp1*, genes associated with more active BAT (Fig. 1A).

We also examined the BAT expression of neuregulin-4 (Nrg4) since this member of the epidermal growth factor family has been shown to be secreted by BAT and to regulate hepatic lipid metabolism in an endocrine fashion (Wang *et al.* 2014). Our results show that cold exposure induced a rapid increased in *Nrg4* mRNA expression in BAT (Fig. 1A).

The increased BAT activity upon cold exposure was also evident by the denser appearance of interscapular BAT in mice kept at 4°C (Fig. 1B). Similar effects were observed in the inguinal WAT depot upon cold exposure (Fig. 1C and D). Cold exposure reduced plasma TG concentrations by 55±6% ($P<0.05$) but had no effect on plasma cholesterol concentrations (Fig. 1E and F).

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Table 2 Effect of 24-h exposure to 4°C on body weight, food intake, blood glucose and adipose tissue weight.

	23°C	4°C
Body weight before (g)	25.0 ± 0.7	26.6 ± 0.8
Body weight after (g)	25.0 ± 0.7	25.7 ± 0.6
Delta body weight (g)	0.0 ± 0.1	−0.9 ± 0.2*
Food intake (g)	3.4 ± 0.2	5.3 ± 0.3*
Relative food intake (mg/g BW)	137 ± 6	206 ± 13*
Blood glucose (mM)	6.1 ± 0.1	6.2 ± 0.5
iBAT weight (mg)	110 ± 8	117 ± 5
Relative iBAT weight (% BW)	0.44 ± 0.03	0.46 ± 0.02
iWAT weight (mg)	200 ± 12	183 ± 8
Relative iWAT weight (%)	0.81 ± 0.06	0.71 ± 0.03
gWAT weight (mg)	367 ± 23	348 ± 21
Relative gWAT weight (%)	1.46 ± 0.07	1.35 ± 0.05

Data from 24-h cold-exposed male C57Bl/6J mice. Values are averages ± S.E.M.; $n = 6$.
*$P < 0.05$ vs. 23°C.
iBAT, interscapular brown adipose tissue; iWAT, inguinal white adipose tissue; gWAT, gonadal white adipose tissue.

Cold exposure increases hepatic triglycerides predominantly in the periportal zone while reducing hepatic glycogen content

Upon 24-h cold exposure, the hepatic TG concentration was significantly increased by 2.2 ± 0.4 fold (Table 3). This accumulation of hepatic TGs did not provoke an elevation in the plasma concentrations of AST and ALT, indicating an absence of actual liver injury (Table 3). Hepatic cholesterol concentrations were not changed by cold exposure. Hepatic glycogen concentrations were reduced by 87 ± 6% ($P < 0.05$), while hepatic glucose-6-phosphate (G6P) concentrations were not affected (Table 3).

We investigated in which hepatic zone(s) TGs accumulated upon cold exposure. Since glutamine synthetase (Gs) is only present in the pericentral zone (Stanulović *et al.* 2007), Gs immunohistochemistry was used to identify this region of the liver. Immunohistochemistry for both Gs and the lipid droplet protein perilipin-2 (Plin2) revealed that 24-h cold exposure resulted in increased Plin2 protein staining predominantly but not exclusively in the periportal zone (Fig. 2).

Cold exposure reduces hepatic lipogenic gene expression

Next, we studied whether the increased hepatic TG content might be the result of reduced fatty acid oxidation and/or increased fatty acid synthesis. For fatty acid oxidation, we determined the expression of genes controlled by the major regulators of fatty acid oxidation, namely the nuclear receptors peroxisome proliferator-activated receptor-α (Pparα) and Pparβ/δ. With respect to Pparα target genes, 24-h cold exposure induced the expression of the

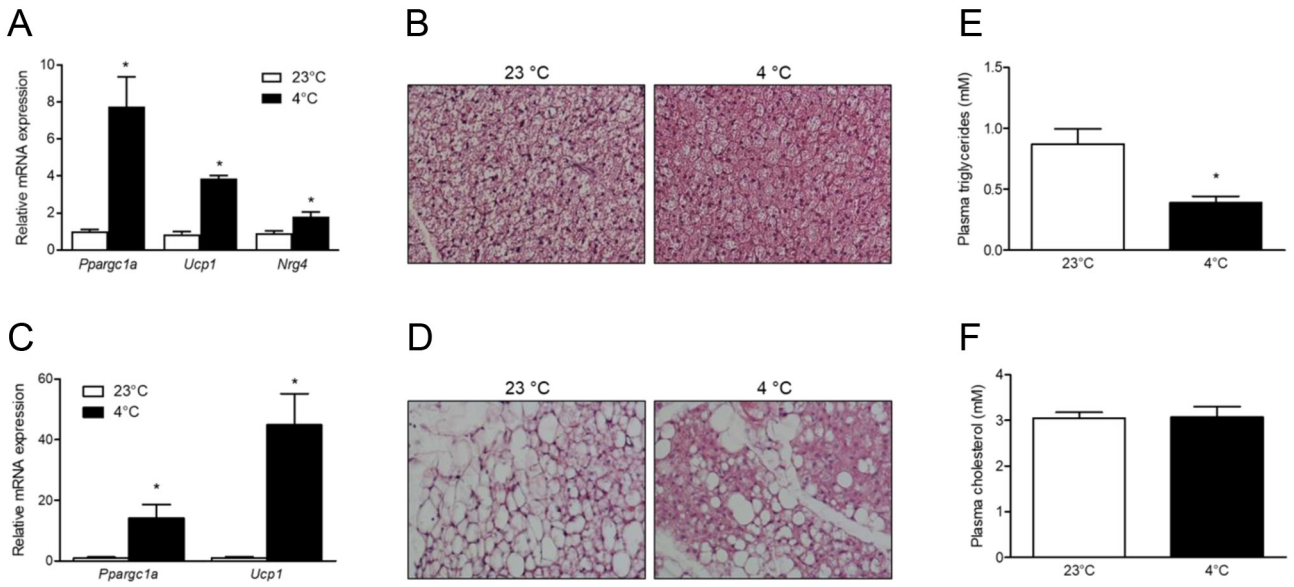

Figure 1
Exposure to 4°C for 24 h activates BAT, induces 'browning' of WAT and reduces plasma TG concentrations. Data from 24-h cold-exposed male C57Bl/6J mice. (A) Relative interscapular BAT mRNA expression normalized to *Rn18s* and *B2m* with data from mice kept at 23°C defined as '1'. (B) Representative H&E staining of the interscapular BAT, original magnification 200×. (C) Relative inguinal WAT mRNA expression normalized to *Rn18s* and *B2m* with data from mice kept at 23°C defined as '1'. (D) Representative H&E staining of the inguinal WAT, original magnification 200×. (E) Plasma TG concentrations. (F) Plasma cholesterol concentrations. Values are averages ± S.E.M.; $n = 6$; *$P < 0.05$ vs. 23°C. Nrg4, neuregulin-4; Ppargc1a, proliferator-activated receptor-gamma co-activator 1a; Ucp1, uncoupling protein-1.

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Table 3 Effect of 24-h exposure to 4°C on liver weight, hepatic biomarkers and hepatic metabolites.

	23°C	4°C
Liver weight (mg)	1247 ± 42	1347 ± 31
Relative liver weight (% BW)	4.98 ± 0.06	5.25 ± 0.03*
Plasma ALT (U/L)	54 ± 16	32 ± 4
Plasma AST (U/L)	79 ± 3	74 ± 8
Hepatic triglycerides (nmol/mg)	8.8 ± 0.8	19.1 ± 3.4*
Hepatic free cholesterol (nmol/mg)	3.7 ± 0.1	3.8 ± 0.2
Hepatic cholesteryl esters (nmol/mg)	1.2 ± 0.2	1.4 ± 0.1
Hepatic glycogen (nmol/mg)	367 ± 74	47 ± 21*
Hepatic G6P (nmol/mg)	0.53 ± 0.08	0.38 ± 0.06

Data from 24-h cold-exposed male C57Bl/6J mice. Values are averages ± S.E.M.; $n = 6$.
*$P < 0.05$ vs. 23°C.
ALT, alanine transaminase; AST, aspartate transaminase; G6P, glucose-6-phosphate.

gene encoding carnitine palmitoyltransferase-1a (Cpt1a) by 1.8 ± 0.3 fold ($P < 0.05$) while decreasing expression of the gene encoding fibroblast growth factor-21 (Fgf21) by 69 ± 10% ($P < 0.05$) (Fig. 3A). The hepatic mRNA expression of the Pparβ/δ target genes encoding for perilipin-5 (Plin5) and ST3 β-galactoside α-2,3-sialyltransferase 5 (St3gal5) was significantly induced upon 24-h exposure to 4°C (Fig. 3B).

Expression of genes involved in hepatic fatty acid synthesis, such as the major lipogenic transcription factor sterol-regulatory element-binding protein-1c (Srebp-1c) and its target genes encoding acetyl-coenzyme A carboxylase alpha (Acaca) and stearoyl-coenzyme A desaturase-1 (Scd1) were all significantly reduced upon cold exposure (Fig. 3C). Since transcription of Srebp-1c and hence its target genes is regulated by liver X receptor (Lxr) (Schultz *et al.* 2000), the reduced hepatic mRNA expression of these genes might reflect reduced Lxr

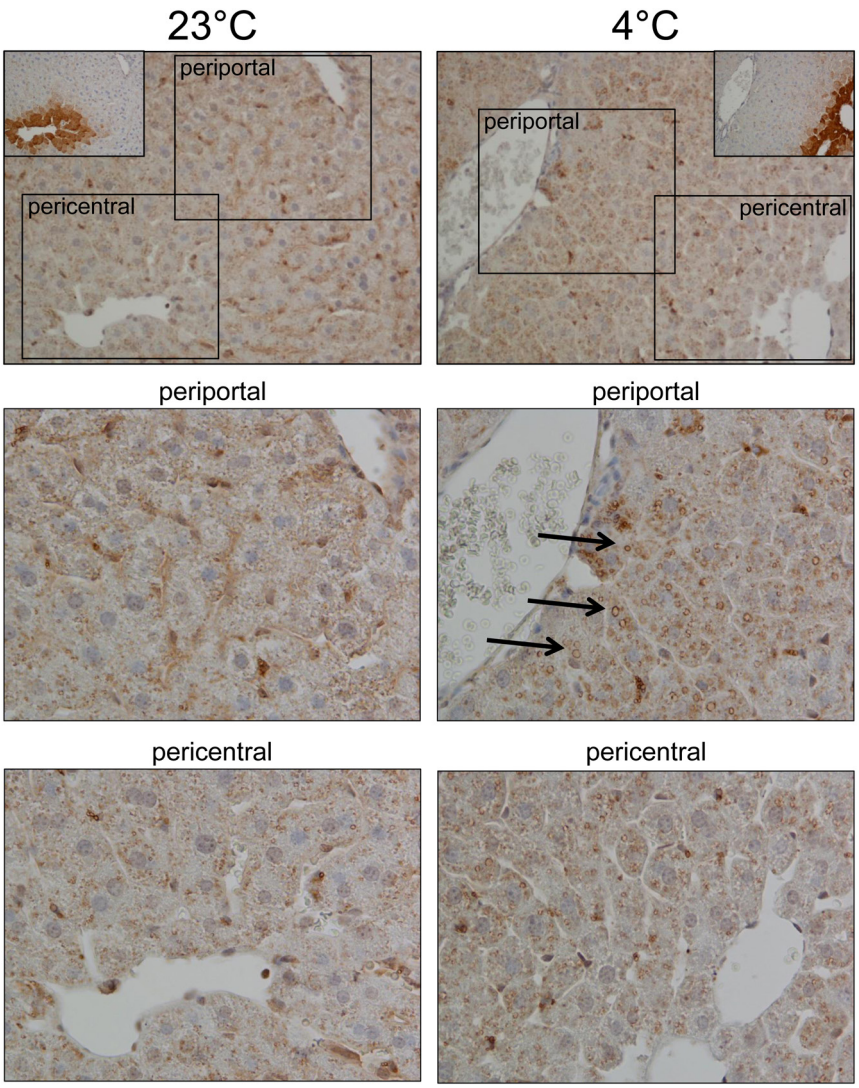

Figure 2
A predominant increase of perilipin-2 protein in the periportal zone of livers from mice exposed to 4°C for 24 h. Representative glutamine synthase (Gs) and perilipin-2 (Plin2) expression in livers of 24-h cold-exposed male C57Bl/6J mice. Top row: Plin2 immunohistochemical staining (original magnification 200×) outlining the locations of the periportal and pericentral pictures. The insets show Gs immunohistochemical staining (original magnification 200×) of the same livers used to localize the periportal and the pericentral zones. Middle row: Plin2 immunohistochemical staining of the periportal zone, original magnification 400×. Arrows indicate examples of lipid droplets with Plin2 protein expression on the surface. Bottom row: Plin2 immunohistochemical staining of the pericentral zone, original magnification 400×.

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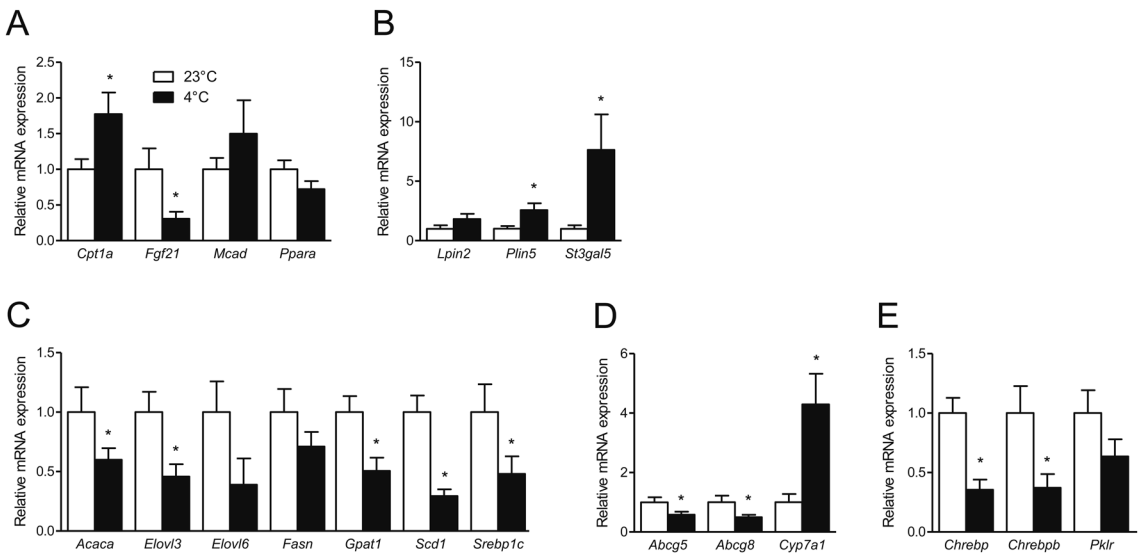

Figure 3

Exposure to 4°C for 24 h reduces hepatic lipogenic gene expression. Relative hepatic mRNA expression of 24-h cold-exposed male C57Bl/6J mice. (A) Expression of Pparα and its target genes. (B) Expression of Pparβ/δ target genes. (C) Expression of genes involved in fatty acid and TG synthesis. (D) Expression of Lxr target genes. (E) Expression of Chrebp and its target genes. Hepatic mRNA expression is normalized to *Rn18s* and *Actb* with data from mice kept at 23°C defined as '1'. Values are averages ± S.E.M.; $n=6$; *$P<0.05$ vs. 23°C. Abcg5, ATP-binding cassette G5; Abcg8, ATP-binding cassette G8; Acaca, acetyl-coenzyme A carboxylase alpha; Chrebp, carbohydrate-response-element binding protein; Cpt1a, palmitoyltransferase-1a; Cyp7a1, cytochrome P450 7A1; Elovl3, Elongation of long-chain fatty acid family member 3; Elovl6, Elongation of long-chain fatty acid family member 6; Fasn, fatty acid synthase; Fgf21, fibroblast growth factor-21; Gpat1, glycerol-3-phosphate acyltransferase-1; Lpin2, lipin-2; Lxr, liver X receptor; Mcad, medium-chain acyl-CoA dehydrogenase; Pklr, pyruvate kinase liver and red blood cells; Plin5, perilipin-5; Pparα; peroxisomal proliferator-activated receptor-α; Pparβ/δ, peroxisomal proliferator-activated receptor-β/δ; Scd1, stearoyl-coenzyme A desaturase-1; Srebp-1c, sterol-regulatory element-binding protein-1c; St3gal5, ST3 β-galactoside α-2,3-sialyltransferase 5.

signaling. Therefore, we also determined the expression of the non-lipogenic Lxr target genes *Abcg5*, *Abcg8* and *Cyp7a1* that encode ATP-binding cassette G5, -G8 and cytochrome P450 7A1, respectively (Fig. 3D). Cold exposure for 24 h significantly reduced hepatic expression of *Abcg5* and *Abcg8* by 42 ± 10% and 50 ± 8%, respectively. In contrast, hepatic *Cyp7a1* mRNA expression was significantly increased by 330 ± 100%.

Most lipogenic genes controlled by Srebp-1c are also regulated by the carbohydrate responsive element-binding protein (Chrebp) (Ishii *et al.* 2004) whose expression is also suggested to be controlled by Lxr (Cha & Repa 2007). Cold exposure strongly reduced hepatic *Chrebp* mRNA expression and the Chrebp target genes *Chrebpb* and *Pklr*, encoding the protein pyruvate kinase liver and red blood cells, albeit that the latter reduction failed to reach statistical significance (Fig. 3E).

Cold exposure increases the hepatic bile acid concentration

Twenty-four hour exposure to 4°C suppressed transcriptional activity of the lipogenic transcription factors Srebp-1c, Lxr and Chrebp, indicative of reduced Lxr activity. In contrast, expression of the Lxr target gene *Cyp7a1* was increased by more than 4-fold. Cyp7a1 is the rate-limiting enzyme in the classical BA synthesis pathway. However, other enzymes also contribute to the classical and the alternative BA synthesis pathway (Russell 2003). We therefore explored the effect of cold exposure on the hepatic mRNA expression of these enzymes and on hepatic, fecal and plasma BAs. Cold exposure significantly reduced the expression of *Cyp27a1* by 44 ± 3% (Fig. 4A), while hepatic BA concentrations and fecal BA excretion were increased 2.8- and 1.3-fold ($P<0.05$ for both), respectively (Fig. 4B and C). Plasma BA concentrations were reduced, but this effect failed to reach significance (Fig. 4D).

Reduced VLDL secretion in cold-exposed mice

Previous studies have demonstrated that both Chrebp and Lxr activity as well as BAs affect VLDL secretion (Grefhorst *et al.* 2002, Elzinga *et al.* 2003, Watanabe *et al.* 2004, Grefhorst & Parks 2009, Wu *et al.* 2015). We therefore also determined the VLDL-TG secretion rates of cold-exposed

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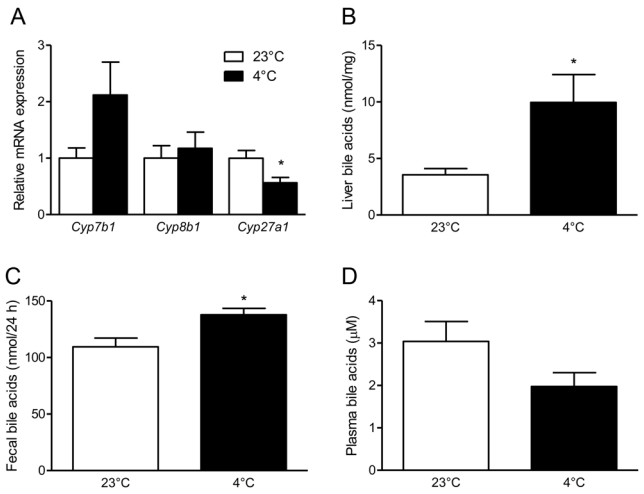

Figure 4
Elevated hepatic bile acids upon exposure to 4°C for 24 h. Data from 24-h cold-exposed male C57Bl/6J mice. (A) Relative hepatic mRNA expression of genes encoding enzymes involved in bile acid (BA) synthesis. Expression is normalized to *Rn18s* and *Actb* with data from mice kept at 23°C defined as '1'. (B) Hepatic BA concentrations. (C) Fecal BA excretions. (D) Plasma BA concentrations. Values are averages ± S.E.M.; $n = 5–6$; *$P < 0.05$ vs. 23°C. Cyp7b1, cytochrome P450 7A1; Cyp8b1, cytochrome P450 8B1; Cyp27a1, cytochrome P450 27A1.

mice using the lipoprotein lipase (Lpl) inhibitor triton WR-1339. VLDL-TG secretion was reduced significantly by approximately 50% after 24 h of cold exposure (Fig. 5A and B). Analysis of the lipid composition of nascent VLDL particles revealed that cold exposure reduced the relative TG content and elevated the relative cholesterol content of the VLDL particles without affecting VLDL particle size (Table 4). The latter is in line with the fact that

Table 4 Effect of 24-h exposure to 4°C on lipid composition and size of nascent VLDL particles.

	23°C	4°C
Triglycerides (% lipid)	74.9 ± 0.7	72.0 ± 1.0*
Phospholipids (% lipid)	15.8 ± 0.6	16.1 ± 0.6
Free cholesterol (% lipid)	8.3 ± 0.3	10.1 ± 0.6*
Cholesteryl esters (% lipid)	0.9 ± 0.2	1.8 ± 0.2*
Calculated diameter (nm)	76.6 ± 2.9	72.3 ± 2.8

Data from nascent VLDL collected from 24-h cold-exposed male C57Bl/6J mice. The mice were fasted for 5 h prior to injection with triton WR-1339. Two hours after injection, blood was collected and VLDL was isolated from plasma by ultracentrifugation. The lipid concentrations are expressed as percentage relative to the total lipid concentration. VLDL diameter was calculated using the formula diameter (nm) = 60 × ((0.211 × TG/PL) + 0.27) in which TG is the relative TG concentration and PL the relative phospholipid concentration. Values are averages ± S.E.M.; $n = 6$.
*$P < 0.05$ vs. 23°C.

cold exposure did not affect the relative apolipoprotein B (ApoB) protein concentration of the VLDL fraction (Fig. 5C). The expression of genes encoding important regulators of VLDL secretion, *e.g.*, ApoB and microsomal TG transfer protein (Mttp), was not affected by cold exposure (Fig. 5D). VLDL-TG secretion is not only suppressed by BAs but also by catecholamines (Rasouli & Zahraie 2006) and thyroid hormone 3,3′,5-triiodothyronine (T3) (Wilcox & Heimberg 1991). Cold exposure did not affect hepatic and plasma catecholamine concentrations (Fig. 6A and 6B) but did increase plasma T3 concentrations by 2.2 ± 0.1 fold ($P < 0.05$) (Fig. 6C). In agreement, cold exposure enhanced hepatic expression of the T3 target gene *Thrsp* (Zilz 1990), encoding thyroid hormone responsive (Fig. 6D).

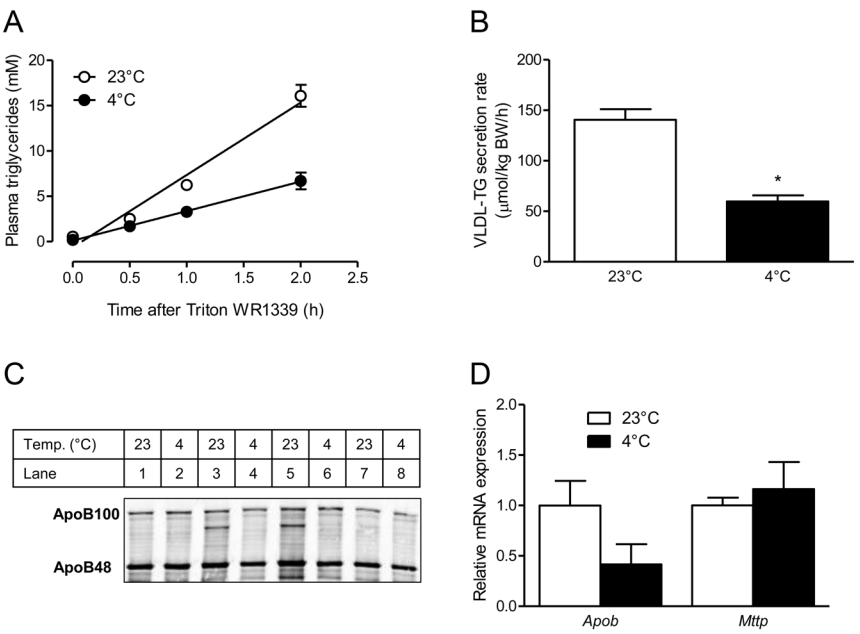

Figure 5
Exposure to 4°C results in a reduced VLDL-TG secretion. Data from 24-h cold-exposed male C57Bl/6J mice. Mice were fasted for 5 h prior to triton WR-1339 injection. (A) Plasma TG concentrations before and at indicated time points after injection of triton WR-1339. (B) VLDL-TG secretion rates calculated from the plasma TG vs. time curve. (C) Immunoblot of apoB in nascent VLDL. For this, blood was collected 2 h after triton WR-1339 and VLDL was isolated from plasma by ultracentrifugation. Equal amounts of TG were loaded per well. (D) Relative hepatic mRNA expression of genes encoding proteins involved in VLDL secretion. Expression is normalized to *Rn18s* and *Actb* with data from mice kept at 23°C defined as '1'. Values are averages ± S.E.M.; $n = 6$; *$P < 0.05$ vs. 23°C. ApoB, apolipoprotein B; Mttp, microsomal TG transfer protein.

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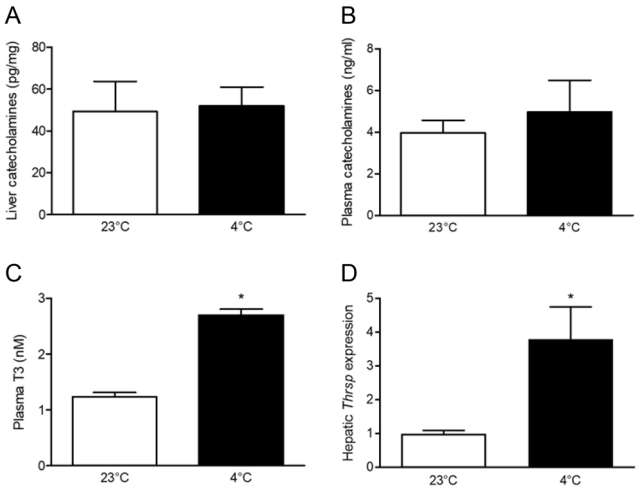

Figure 6
Exposure to 4°C for 24 h results in elevated plasma T3 concentrations. Data from 24-h cold-exposed male C57Bl/6J mice. (A) Hepatic catecholamine (norepinephrine and epinephrine) contents. (B) Plasma catecholamine (norepinephrine and epinephrine) concentrations. (C) Plasma 3,3′,5-triiodothyronine (T3) concentrations. (D) Relative hepatic *Thrsp* mRNA expression encoding thyroid hormone responsive. Expression is normalized to *Rn18s* and *Actb* with data from mice kept at 23°C defined as '1'. Values are averages ± S.E.M.; $n=6$; *$P<0.05$ vs. 23°C.

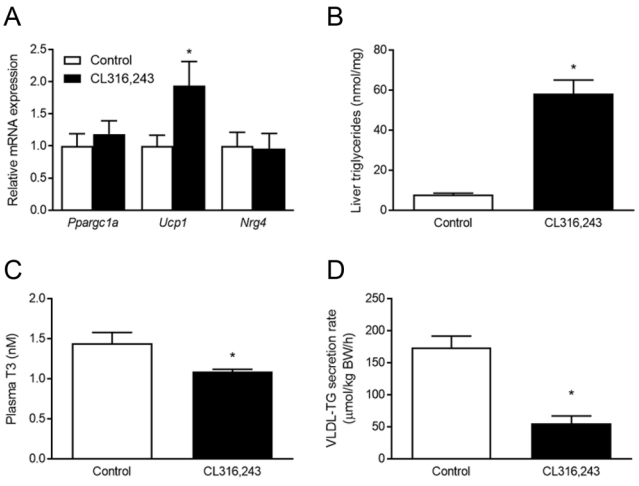

Figure 7
Treatment with the β3-adrenoreceptor agonist CL316,243 stimulates BAT, induces hepatic TG concentrations and reduces VLDL-TG secretion despite reduced plasma T3 concentrations. Data from male mice treated with or without 3 times 1 mg CL316,243 per kg bodyweight. (A) Relative interscapular BAT mRNA expression normalized to *Rn18s* and *B2m* with data from control mice defined as '1'. (B) Hepatic TG concentrations. (C) Plasma 3,3′,5-triiodothyronine (T3) concentrations. (D) VLDL-TG secretion rates calculated from the plasma TG *vs.* time curve from samples collected after injection of Triton WR-1339. Values are averages ± S.E.M.; $n=6$; *$P<0.05$ vs. control mice. Nrg4, neuregulin-4; Ppargc1a, proliferator-activated receptor-gamma co-activator 1a; Ucp1, uncoupling protein-1.

To investigate the link between activated BAT, increased plasma T3 and reduced VLDL-TG secretion in more detail, we activated BAT by treating mice with the β3-adrenoreceptor agonist CL316,243. As expected, CL316,243 treatment enhanced BAT activity, as reflected by the increased BAT *Ucp1* mRNA expression (Fig. 7A). In contrast to cold exposure, CL316,243 treatment did not increase BAT *Ppargc1a* and *Nrg4* mRNA expression. CL316,243-treatment also did not affect hepatic mRNA expression of the studied genes, apart from inductions in *Elovl6*, *Cyp7a1* and *Apob* (Table 5). Treatment with the β3-adrenoreceptor agonist resulted in a massive hepatic TG accumulation from 7.9 ± 0.7 in control mice to 58.3 ± 6.7 nmol/mg in CL316,243-treated mice ($P<0.05$) (Fig. 7B). In contrast to cold exposure, CL316,243 treatment reduced plasma T3 concentrations (Fig. 7C). Nevertheless, CL316,243-activation of BAT still coincided with a strongly reduced VLDL-TG secretion (Fig. 7D).

Remodeling of hepatic cholesterol metabolism upon cold exposure

Our results so far suggest that cold exposure stimulated hepatic BA synthesis, which might affect cholesterol concentrations. However, hepatic cholesterol concentrations were not affected, suggesting changes in the regulation of cellular cholesterol homeostasis in cold-exposed mice. Therefore, we investigated the transcriptional activity of sterol-regulatory element-binding protein-2 (Srebp-2), the major regulator of hepatic cholesterol concentrations (Goldstein *et al.* 2006, Radhakrishnan *et al.* 2008). Although hepatic mRNA expression of *Srebp2* was not significantly affected by 24-h cold exposure, the mRNA expression of the Srebp-2 target genes *Hmgcr*, *Ldlr* and *Pcsk9* (encoding 3-hydroxy-3-methylglutaryl-CoA reductase, LDLR and proprotein convertase subtilisin/kexin type 9, respectively) were significantly upregulated (Fig. 8).

Preserved effects of cold exposure on hepatic lipogenic gene expression

To study whether the changes in hepatic gene expression persisted upon prolonged cold exposure, mice were kept at 28°C or 4°C for 10 days. During the cold exposure, the plasma TG concentrations declined (one-way ANOVA: $F(4,72)=0.932$, $P<0.05$) and remained reduced after 10-day exposure to 4°C (Fig. 9A). Also upon prolonged cold exposure, the mRNA expression levels of genes reflecting BAT activity were increased (Fig. 9B). Likewise, prolonged cold exposure resulted in a modest but statistically significant elevation of the hepatic TG content, while

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Table 5 Effect of treatment with the β3-adrenoreceptor agonist CL316,243 on hepatic gene expression.

	Control	CL316,243
Pparα and its target genes		
Cpt1a	1.00±0.23	1.60±0.56
Fgf21	1.00±0.18	4.72±2.49
Mcad	1.00±0.18	4.22±2.08
Ppara	1.00±0.29	2.43±1.68
Pparβ/δ target genes		
Lpin2	1.00±0.19	1.96±0.74
Plin5	1.00±0.33	1.58±0.57
St3gal5	1.00±019	1.38±0.46
Fatty acid and TG synthesis		
Acaca	1.00±0.15	1.49±0.17
Elovl3	1.00±0.18	0.93±0.15
Elovl6	1.00±0.15	1.93±0.38*
Fasn	1.00±0.35	0.94±0.50
Gpat1	1.00±0.18	1.65±0.55
Scd1	1.00±0.19	0.56±0.28
Srebp1c	1.00±0.27	0.69±0.07
Chrebp and its target genes		
Chrebp	1.00±0.19	0.57±0.15
Chrebpb	1.00±0.12	0.70±0.14
Pklr	1.00±0.28	0.85±0.15
Lxr target genes		
Abcg5	1.00±0.15	1.34±0.41
Abcg8	1.00±0.24	1.45±0.33
Cyp7a1	1.00±0.13	2.06±0.33*
BA synthesis		
Cyp7b1	1.00±0.24	0.86±0.21
Cyp8b1	1.00±0.25	0.95±0.21
Cyp27a1	1.00±0.33	0.97±0.17
VLDL secretion		
Apob	1.00±0.08	2.25±0.63
Mttp	1.00±0.19	3.35±1.88
T3 target gene		
Thrsp	1.00±0.14	0.73±0.05

Hepatic gene expression of male mice treated with or without 3 times 1 mg CL316,243 per kg bodyweight. Results were normalized to *Rn18s* and *Actb* mRNA expression with data from control mice defined as '1'. Values are averages±S.E.M.; $n=6$.
*$P<0.05$ vs. control mice.

hepatic cholesterol concentrations were not affected (Fig. 9C). Hepatic mRNA expression analysis showed that prolonged cold reduced mRNA expression of the Pparα target genes *Fgf21* and *Mcad* by 97.9±0.2% and 53±8%, respectively (Fig. 9D). Also the Pparβ/δ target genes *Lpin2* and *St3gal5* and most Srebp-1c and Chrebp target genes were reduced by prolonged cold exposure (Fig. 9E and G). While *Cyp7a1* mRNA expression was not affected by 10 days of 4°C (Fig. 9H), it increased the expression of *Cyp7b1* and *Cyp8b1* (Fig. 9I). In addition, we found that 10 days of 4°C reduced hepatic *Apob* mRNA expression and had no effect on hepatic *Mttp* mRNA expression (Fig. 9J). Of the Srebp-2 target genes, only *Hmgcr* mRNA expression was affected by exposure to 4°C for 10 days (Fig. 9K).

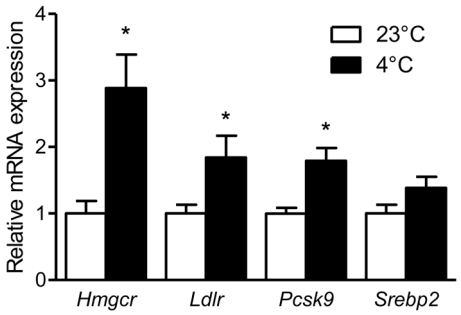

Figure 8
Modulation of Srebp-2 signaling in livers of mice exposed to 4°C for 24 h. Relative hepatic mRNA expression of Srebp-2 and its target genes of 24-h cold-exposed male C57Bl/6J mice. Expression is normalized to *Rn18s* and *Actb* with data from mice kept at 23°C defined as '1'. Values are averages±S.E.M.; $n=6$; *$P<0.05$ vs. 23°C. Hmgcr, 3-hydroxy-3-methylglutaryl-CoA reductase; Ldlr, LDL receptor; Pcsk9, proprotein convertase subtilisin/kexin type 9; Srebp-2, sterol-regulatory element-binding protein-2.

Discussion

Cold exposure is a common strategy to activate BAT in laboratory animals to determine the potentially positive effects of activated BAT on metabolism. Here, we describe that exposure to 4°C for 24 h has multiple, likely intertwined effects on the liver: it increases hepatic TG concentrations, reduces hepatic glycogen content, reduces hepatic lipogenic gene expression, lowers VLDL-TG secretion, stimulates BA synthesis, induces hepatic BA concentrations and modulates hepatic cholesterol homeostasis.

As has been reported in multiple studies (Bartelt *et al.* 2011, Khedoe *et al.* 2015, van den Beukel *et al.* 2015), activation of BAT by cold exposure reduces plasma TG concentrations. Although a recent study reported that the cold-induced decline in plasma TG is transient (Flachs *et al.* 2017), we found a persistent reduction in plasma TG during the 10-day cold exposure. An explanation for this difference between our study and the previous one could be the housing conditions. We choose to house the mice individually to prevent them from keeping each other warm, while Flachs *et al.* caged the mice in groups of 3–4 which might have resulted in a significantly reduced individual exposure to low temperatures (Flachs *et al.* 2017).

An increased hepatic TG content can be caused by increased hepatic fatty acid uptake, decreased fatty acid oxidation, increased hepatic fatty acid and TG synthesis, and/or decreased VLDL-TG secretion (Willebrords *et al.* 2015). Since cold exposure enhances WAT lipolysis (Himms-Hagen 1972), it is expected that this experimental

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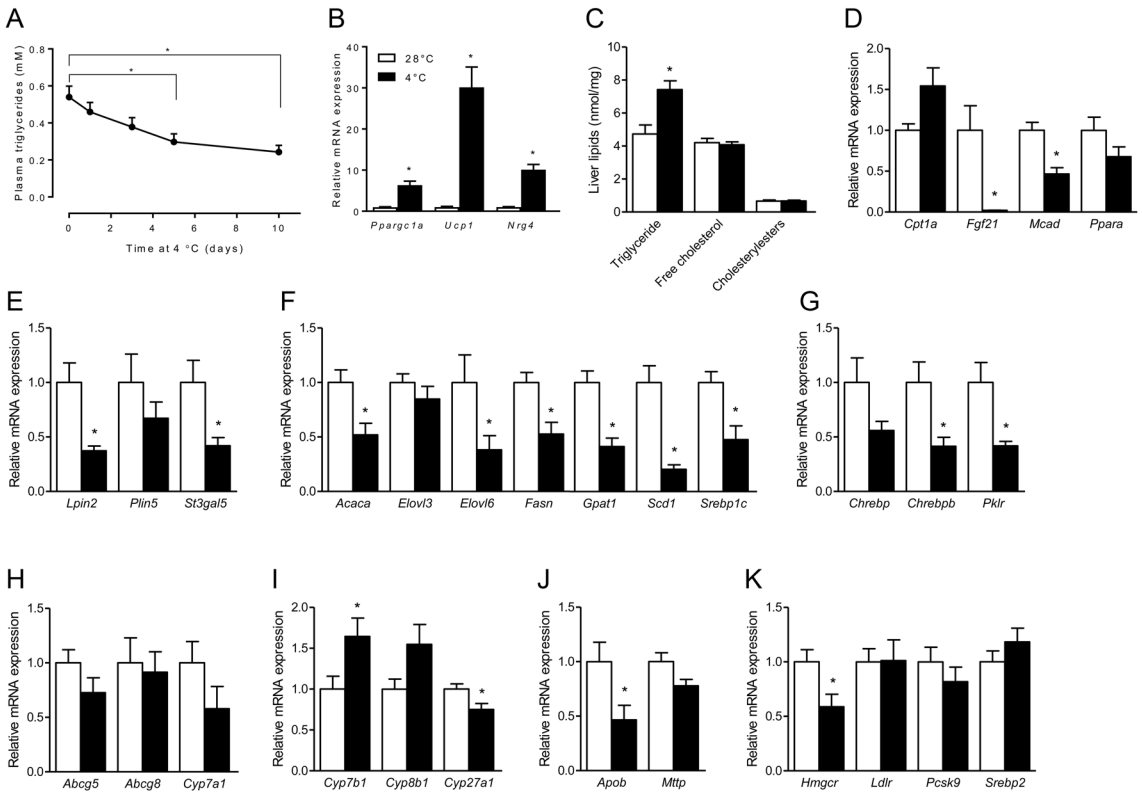

Figure 9

Increased hepatic TG content and lower hepatic lipogenic gene expression persist after 10 days of exposure to 4°C. Data from 10-day cold-exposed male C57Bl/6J mice. (A) Plasma TG concentrations during the 10-day exposure to 4°C. Values are averages ± S.E.M.; $n = 9–19$; $^*P < 0.05$ in Tukey's post hoc test after one-way ANOVA. (B) Relative interscapular BAT mRNA expression normalized to *Rn18s* and *B2m* with data from mice kept at 28°C defined as '1'. (C) Hepatic TG and cholesterol concentrations. (D) Relative hepatic mRNA expression of Pparα and its target genes. (E) Relative hepatic mRNA expression of Pparβ/δ target genes. (F) Relative hepatic mRNA expression of genes involved in fatty acid and TG synthesis. (G) Expression of Chrebp and its target genes. (H) Expression of Lxr target genes. (I) Relative hepatic mRNA expression of genes encoding enzymes involved in bile acid (BA) synthesis. (J) Relative hepatic mRNA expression of genes encoding proteins involved in VLDL secretion. (K) Relative hepatic mRNA expression of Srebp-2 and its target genes. Hepatic expression is normalized to *Rn18s* and *Actb* with data from mice kept at 28°C defined as '1'. Values are averages ± S.E.M.; $n = 8–9$; $^*P < 0.05$ vs. 28°C. Abcg5, ATP-binding cassette G5; Abcg8, ATP-binding cassette G8; Acaca, acetyl-Coenzyme A carboxylase alpha; ApoB, apolipoprotein B; Chrebp, carbohydrate-response-element binding protein; Cpt1a, palmitoyltransferase-1a; Cyp7a1, cytochrome P450 7A1; Cyp7b1, cytochrome P450 7A1; Cyp8b1, cytochrome P450 8B1; Cyp27a1, cytochrome P450 27A1; Elovl3, Elongation of long-chain fatty acid family member 3; Elovl6, Elongation of long-chain fatty acid family member 6; Fasn, fatty acid synthase; Fgf21, fibroblast growth factor-21; Gpat1, glycerol-3-phosphate acyltransferase-1; Hmgcr, 3-hydroxy-3-methylglutaryl-CoA reductase; Ldlr, low-density lipoprotein receptor; Lpin2, lipin-2; Lxr, liver X receptor; Mcad, medium-chain acyl-CoA dehydrogenase; Mttp, microsomal TG transfer protein; Nrg4, neuregulin-4; Pcsk9, proprotein convertase subtilisin/kexin type 9; Pklr, pyruvate kinase liver and red blood cells; Plin5, perilipin-5; Ppargc1a, proliferator-activated receptor-gamma co-activator 1a; Pparα, peroxisomal proliferator-activated receptor-α; Pparβ/δ; peroxisomal proliferator-activated receptor-β/δ; St3gal5, ST3 β-galactoside α-2,3-sialyltransferase 5; Scd1, stearoyl-coenzyme A desaturase-1; Srebp-1c, sterol-regulatory element-binding protein-1c; Srebp-2, sterol-regulatory element-binding protein-2; Ucp1, uncoupling protein-1.

strategy will also result in an increased fatty acid flux from WAT to the liver. Indeed, we found that cold exposure increased the hepatic expression of genes regulated by PPARβ/δ, a transcription factor activated by fatty acids derived from endogenous sources such as adipose tissue (Sanderson *et al.* 2009), reflecting increased hepatic fatty acid uptake. Moreover, we found that 24-h cold exposure resulted in a predominant but not exclusive accumulation of lipids in the periportal zone, the zone where fatty acid uptake and oxidation are mainly localized (Hijmans *et al.* 2014). Thus, a periportal accumulation also points toward an increased fatty acid uptake and/or reduced fatty acid oxidation. The latter seems not very likely, at least at the level of expression of genes encoding rate-limiting enzymes in fatty acid oxidation, such as *Cpt1a* and *Mcad*. In line, it has been shown that 10 days of cold exposure did not reduce but rather increased hepatic fatty acid oxidation in rats, likely in an attempt to enhance ATP production (Iossa *et al.* 1994).

Both short- and long-term cold exposure resulted in marked reductions in hepatic lipogenic gene expression. Thus, an increased hepatic fatty acid and TG synthesis

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is likely not responsible for the increased hepatic TG content. Reduced lipogenesis has been shown before in liver slices from rats exposed to cold that had a reduced ability to convert ^{14}C-acetate into ^{14}C-fatty acids (Masoro *et al.* 1957). Our data show that the reduced hepatic lipogenesis is the result of the reduced activities of the three major lipogenic transcription factors: Srebp-1c, Lxr and Chrebp. Although it has previously been shown that 4 h exposure to cold reduced hepatic *Srebp-1c* mRNA expression by 1.7-fold in mice (Goetzman *et al.* 2005), we here also show an effect on Chrebp signaling. The hepatic glycogen concentrations did drop by almost 90% upon 24 h cold exposure, which is in line with a previous study (Bobbioni-Harsch *et al.* 1994). This reduction in glycogen is likely the result of an increased hepatic glucose production necessary to sustain the increased whole-body energy demand to increase (non) shivering thermogenesis as suggested previously (Cunningham *et al.* 1985). It is to be expected that the elevated hepatic glucose production will result in a reduced flux through the pentose-5-phosphate pathway and hence a lower concentration of xylulose-5-phosphate. Both G6P and xylulose-5-phosphate are considered drivers of Chrebp transcriptional activity (Dentin *et al.* 2012). Thus, cold exposure increases hepatic glucose production, likely resulting in a reduced transcriptional activity of Chrebp.

A direct link between stimulation of BAT by cold exposure and the reduced hepatic lipogenic gene expression might be established by increased Nrg4 concentrations. Mice deficient for Nrg4 have elevated hepatic lipogenic gene expression due to enhanced translocation of Srebp-1c to the nucleus, likely due to higher Lxr activity (Wang *et al.* 2014). Our results show that cold exposure elevates BAT *Nrg4* mRNA expression. Thus, the reduced hepatic Lxr-Srebp-1c transcriptional activity in cold-exposed mice could be due to increased secretion of Nrg4 by BAT.

A remarkable finding was the severe reduction of hepatic *Fgf21* mRNA expression upon cold exposure for either 24 h or 10 days. Numerous factors have been reported to regulate *Fgf21* mRNA expression, as recently reviewed (Strowski 2017). The reduced *Fgf21* mRNA expression in our cold-exposed mice might be a reflection of the reduced transcriptional activity of Chrebp since this factor is among the regulators of *Fgf21* transcription, at least in rat hepatocytes (Iizuka *et al.* 2009).

Exposure to 4°C for 24 h resulted in a severe reduction of VLDL-TG secretion. While cold exposure has been shown to reduce VLDL-TG secretion in rats (McBurney & Radomski 1969), to the best of our knowledge, the present study and our previous one (van den Beukel *et al.* 2015) are the only ones addressing effects of cold exposure on VLDL-TG secretion in mice. The reduced VLDL-TG secretion may contribute to the increased hepatic TG concentrations in cold-exposed mice. In 1969, McBurney and Radomski (1969) concluded from their experiments with rats that cold exposure resulted in a reduced utilization of fatty acids by the liver for VLDL production, which might result in elevated fatty acid storage as TG in the liver. Altogether, both increased fatty acid uptake and reduced VLDL-TG secretion likely contribute to the increased hepatic TG content in the cold-exposed mice.

VLDL-TG secretion has been reported to be reduced by BAs (Elzinga *et al.* 2003, Watanabe *et al.* 2004) and T3 (Wilcox & Heimberg 1991). Thus, the increased hepatic BA and plasma T3 concentrations in cold-exposed mice are in line with the observed reduced VLDL-TG secretion. Our data show that 24-h exposure to 4°C increased hepatic *Cyp7a1* mRNA expression, suggesting an upregulation of the classical BA synthesis pathway and explaining the increased hepatic BA concentrations. Prolonged exposure to 4°C resulted in increased hepatic *Cyp7b1* and *Cyp8b1* mRNA expression, suggesting a switch from the classical to the alternative BA synthesis pathway when cold exposure persists. This latter observation is in agreement with a recent publication of Worthmann *et al.* (Worthmann *et al.* 2017) who showed that 7-day exposure of male mice to 6°C increased hepatic *Cyp7b1* and *Cyp8b1* but not *Cyp7a1* mRNA expression. In contrast, Shore *et al.* (Shore *et al.* 2013) reported a 50- to 100-fold downregulation of hepatic *Cyp7b1*, *Cyp8b1* and *Cyp7a1* mRNA expression in female mice upon exposure to 8°C for 24 h. The difference in sex of the mice may explain these conflicting results. Female mice are known to have lower hepatic *Cyp7b1* and *Cyp8b1* mRNA expression (Zhang & Klaassen 2010) and higher hepatic *Cyp7a1* mRNA expression (Lu *et al.* 2013). Studies in both male and female mice are warranted to identify potential sex differences in BA metabolism upon cold exposure.

T3 has been suggested to be a factor secreted by activated BAT (reviewed in (Villarroya *et al.* 2017)) due to elevated BAT deiodinase-2 activity. Deiodinase-2 is important for the conversion of 3,3′,5,5′-tetraiodothyronine (T4) into T3 and can be stimulated by BAs (Watanabe *et al.* 2006). The hypothalamic–pituitary–thyroid axis is stimulated by cold (Sotelo-Rivera *et al.* 2014) and T3 has been shown to induce hepatic *Cyp7a1* mRNA expression (Bonde *et al.* 2012); thus, the increased hepatic BA concentrations could also be the result of the increased plasma T3 concentrations. The link between T3 and BA metabolism

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and their (combined) effect on lipid metabolism upon cold exposure require more detailed research. Since T3 has also been reported to suppress VLDL-TG secretion (Wilcox & Heimberg 1991), it might be that the increased plasma T3 in cold-exposed mice attributed to a lower VLDL-TG secretion. However, activation of BAT with CL316,243 reduced VLDL-TG secretion but did not elevate the plasma T3 concentrations. This suggests that the changed plasma T3 concentration in cold-exposed mice is likely not the sole factor reducing VLDL-TG secretion.

Catecholamines might also regulate VLDL-TG secretion, although published data are conflicting. Hepatic denervation has been shown to result in a 99% reduction of hepatic catecholamine content and to enhance VLDL-TG secretion (Rasouli *et al.* 2012), while others found that selective denervation of the sympathetic input toward the liver resulted in decreased VLDL secretion in 19-h fasted but not in 4-h fasted rats (Bruinstroop *et al.* 2013). In our studies, an effect of catecholamines on VLDL-TG secretion upon cold exposure can be ruled out since both hepatic and plasma catecholamine concentrations were not affected. This is in line with a previous observation that exposure to 4°C for 3–6 h did not increase norepinephrine turnover in livers of rats (Teramura *et al.* 2014).

An intriguing observation was that 24-h exposure to 4°C increased hepatic Srebp-2 signaling. Since Srebp-2 transcriptional activity is negatively regulated by ER cholesterol concentrations (Goldstein *et al.* 2006, Radhakrishnan *et al.* 2008), it can be speculated that cold exposure resulted in a reduced ER cholesterol content. Interestingly, ER cholesterol content not only controls Srebp-2 activity but also is the source for VLDL cholesterol: a reduction in hepatic cholesterol content is associated with decreased VLDL secretion (Khan *et al.* 1990). Thus, a reduced ER cholesterol content might have contributed to the reduced VLDL secretion in the cold-exposed mice. This effect was likely temporarily since Srebp-2 signaling was normalized after 10-day exposure to 4°C.

In conclusion, we show that cold exposure has multiple, likely intertwined effects on the liver. Exposure to 4°C for 24 h resulted in elevated hepatic TG concentrations, almost diminished hepatic glycogen content, reduced hepatic lipogenic gene expression, reduced VLDL secretion and higher BA synthesis and hepatic BA concentrations. These effects of cold exposure on the liver should be taken into account when studying effects of cold exposure on metabolism since the liver is the main metabolic organ controlling whole-body TG, cholesterol and BA metabolism.

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

A G, S K, A P N T and J A V made contributions to the conception and design of the experiments. A G, J C B, W D and J S performed the research and were involved in acquisition of the data. A G, J S, G J V, S L, T J V and E C H F analyzed and interpreted the data. A G drafted the manuscript. All authors were involved in critical revision of the manuscript. All authors approved the final version of the manuscript.

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