



Modulation of cAMP levels by high fat diet and curcumin and regulatory effects on CD36/FAT scavenger receptor/fatty acids transporter gene expression

Journal:	<i>BioFactors</i>
Manuscript ID	BIOF-16-0076.R1
Wiley - Manuscript type:	Original Article
Date Submitted by the Author:	n/a
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Keywords:	cAMP, curcumin, CD36, signaling, gene expression

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3 **Modulation of cAMP levels by high fat diet and curcumin and regulatory effects on**
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5 **CD36/FAT scavenger receptor/fatty acids transporter gene expression**
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27 Word count of body: 9504

28 Word count of abstract: 226

29 Number of figures: 6

30 Number of supplemental figures: 4

31 Running title: Modulation of CD36 expression by curcumin
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43 **Abbreviations:** **cAMP**, cyclic adenosine monophosphate; **Cur**, curcumin; **HFD**, high fat diet; **Ldlr**, low
44 density lipoprotein receptor; **LDL**, low density lipoprotein
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Abstract

Curcumin, a polyphenol from turmeric (*Curcuma longa*), reduces inflammation, atherosclerosis and obesity in several animal studies. In *Ldlr*^{-/-} mice fed a high fat diet (HFD), curcumin reduces plasma lipid levels therefore contributing to a lower accumulation of lipids and to reduced expression of fatty acid transport proteins (CD36/FAT, FABP4/aP2) in peritoneal macrophages. In the present study we analyzed the molecular mechanisms by which curcumin (500, 1000, 1500 mg/kg diet, for 4 months) may influence plasma and tissue lipid levels in *Ldlr*^{-/-} mice fed a HFD. In liver, HFD significantly suppressed cAMP levels, and curcumin restored almost normal levels. Similar trends were observed in adipose tissues, but not in brain, skeletal muscle, spleen and kidney. Treatment with curcumin increased phosphorylation of CREB in liver, what may play a role in regulatory effects of curcumin in lipid homeostasis. In cell lines, curcumin increased the level of cAMP, activated the transcription factor CREB and the human CD36 promoter via a sequence containing a consensus CREB response element. Regulatory effects of HFD and Cur on gene expression were observed in liver, less in skeletal muscle and not in brain. Since the cAMP/protein kinase A (PKA)/CREB pathway plays an important role in lipid homeostasis, energy expenditure and thermogenesis by increasing lipolysis and fatty acid β -oxidation, an increase in cAMP levels induced by curcumin may contribute to its hypolipidemic and anti-atherosclerotic effects.

Keywords: CREB, NRF2, PPAR γ , CD36, curcumin, cAMP signaling, gene expression, fat accumulation, fat metabolism, obesity, atherosclerosis, inflammation

Introduction

Curcumin (Cur) is a polyphenol derived from the rhizome of turmeric (*Curcuma longa*), which is traditionally used as spice and for prevention and therapy of many diseases in Asian countries. Cur affects a number of cellular functions in cell culture as well as in animal models and human clinical trials (reviewed in (1-5)). In animal studies, Cur lowers the plasma lipid levels and reduces obesity and atherosclerosis (6-10). Thus, some of the lipid-lowering effects of Cur in plasma might be responsible for its beneficial effects against several conditions in which hyperlipidemia plays important roles, such as inflammation, diabetes, obesity, and atherosclerosis.

Several mechanisms have been proposed to explain the hypolipidemic effects of Cur (reviewed in (11)); however, the molecular mechanisms are not yet completely clear and, as outlined below, it may involve several regulatory pathways. We and others have reported that Cur activates AMP activated kinase (AMPK) in adipocytes and myotubes, the master switch in energy metabolism and fatty acid oxidation (6, 12). Hepatic steatosis is also attenuated by Cur through activation of AMPK (13, 10). In cultured monocytes/macrophages, Cur enhances the activity of the forkhead box O3a (FOXO3a) transcription factor by increasing its phosphorylation and nuclear localization, leading to induction of the expression of FOXO3a-target genes (e.g. sterol carrier protein, 3-hydroxy-3-methylglutaryl-CoA synthase, CD36, FABP4/aP2, MnSOD, catalase, eNOS) relevant for lipid accumulation and resistance to oxidative and lipid-mediated stress (8). Interestingly, Cur increases lipid levels in monocytes/macrophages in cultured cells, but it decreases lipid levels in peritoneal macrophages of *Ldlr*^{-/-} mice supplemented with Cur, suggesting that *in vivo* uptake and metabolism of Cur is different and may play a role in lipid homeostasis (8, 14, 15). Accordingly, the fatty acid transport proteins, CD36/FAT and fatty acid binding protein 4 (FABP4/aP2), are induced by Cur in cultured THP-1 monocytes/macrophages, whereas in peritoneal macrophages of *Ldlr*^{-/-} mice their expression is suppressed after Cur supplementation (8). Based on these results, we have proposed a model in which the hypolipidemic effect of Cur leads to a lower exposure of peritoneal macrophages to plasma lipids, which in turn down-regulates CD36 and aP2 with consequent lower lipid accumulation in these cells (8).

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3 The plasma lipid level is predominately influenced by the interplay of liver, skeletal muscle and
4 adipose tissues. In these tissues, the cAMP/PKA/CREB signaling pathway plays important roles in lipid
5 homeostasis by increasing PKA-mediated lipolysis and fatty acid oxidation (16, 17). In the liver,
6 cAMP/PKA regulates lipid homeostasis by modulating energy expenditure and thermogenesis, by
7 decreasing liver VLDL secretion and increasing lipolysis and fatty acid oxidation (18-21). In
8 skeletal/cardiac muscle, cAMP stimulates CD36-mediated lipid uptake, β -oxidation and mitochondrial
9 biogenesis (22, 23), and protects against muscle atrophy by inhibiting the ubiquitin-proteasome system
10 (24). In skeletal muscle, lipid uptake is stimulated by CD36 membrane exposition and controlled by
11 FOXO1 by yet unclear molecular mechanisms (25-27). In adipocytes as well as in atherosclerotic lesions,
12 increased cAMP decreases lipid content (18). Thus, as described recently for the polyphenol resveratrol
13 (28), up-regulation of tissue cAMP levels by Cur could be at the basis of its hypolipidemic and anti-
14 atherosclerotic effects and similar to other nutritional supplements such as forskolin or caffeine, it may
15 also explain its effect on weight loss (21, 6, 29).
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32 In this study using cell culture and *in vivo* animal models we have investigated whether the
33 induction of cAMP levels in different tissues and subsequent stimulation of lipid transporters and
34 catabolic genes by Cur could be at the basis of its hypolipidemic effects. As an *in vivo* experimental
35 model we used the *Ldlr*^{-/-} mouse which is a good animal model of high fat-induced accelerated
36 hyperlipidemia and atherosclerosis, since when fed a high fat Western style diet, these mice show a high
37 concentration of circulating cholesterol and develop lipid-mediated stress (accumulation of lipids,
38 inflammation, increased oxidative stress) at 8 weeks (30). Our results suggest that a regulatory role of Cur
39 on cAMP-mediated signaling and gene expression may contribute to its effects on prevention of
40 atherosclerosis and inflammation.
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53 **Materials and Methods**

54 **Materials**

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3 Curcumin (Cur), curcuminglucuronide (CurG) , curcuminsulphate (CurS), demethoxycurcumin (DCur),
4
5 and bisdemethoxycurcumin (BDCur) (Toronto Research Chemicals, Inc., Canada), and
6
7 tetrahydrocurcumin (THC) (kindly provided by V. Badamov, Sbinsa Corporation, Piscataway, NJ), were
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9 dissolved in ethanol as 20 mM stock solution. The adenylate cyclase activators, forskolin (20 mM) and
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11 2'-5'-dideoxyadenosine (50 mM), or the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine
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13 (IBMX) (500 mM) (Sigma-Aldrich, St Louis, MI), were dissolved in ethanol and diluted with ethanol
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15 upon usage.
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20 21 **Cell culture and treatments**

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23 The human acute monocytic leukemia cell line THP-1 (TIB-202, American Type Culture Collection
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25 (ATCC)) was cultured in RPMI 1640 medium, 10% fetal calf serum, 2 mmol/L L-glutamine, 1.0 mmol/L
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27 sodium pyruvate, 4.5 g/L glucose containing 100 µg/ml streptomycin and 100 U penicillin. Human
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29 embryonic kidney 293 (HEK293) cells (CRL-1573, ATCC) were grown in Dulbecco's modified Eagle's
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31 medium, 10% fetal calf serum, and 2 mmol/L L-glutamine containing 100 µg/mL streptomycin and 100
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33 U/mL penicillin. THP-1 monocytes (1×10^6 /6 well plate) and HEK293 cells (0.3×10^6 /6 well plate) were
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35 plated 24 h before treatments with compounds.
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38 39 40 **Animal feeding protocol**

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42 $Ldlr^{-/-}$ C57BL/6 male mice (Jackson Laboratory, Bar Harbor, ME) were individually housed at the Jean
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44 Mayer Human Nutrition Research Center on Aging (HNRCA) Comparative Biology Unit at Tufts
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46 University. The mice were kept on a 12 h light/dark cycle and had free access to food and water. After
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48 acclimation for one week, 8 weeks old mice were randomly assigned to five groups of 24 mice each. One
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50 group of mice was fed an AIN-93M diet (Harlan Teklad, TF.08485) for 16 weeks and served as "low fat
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52 diet (LFD) negative controls." The other 4 groups of mice were fed a Western style high fat/cholesterol
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54 AIN-93M diet (HFD) (Harlan Teklad TD.09346) formulated to contain 0.157% cholesterol and 21% fat
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3 by weight for 4 months. AIN-93 mineral and vitamin mixes were used in the formulation of diets. From
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5 the HFD-fed mice, one group of mice was designated as positive HFD control, and the diets of the other
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7 three groups were either supplemented with a low (500 mg/kg), medium (1000 mg/kg) and high (1500
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9 mg/kg) dose of Cur for 16 weeks. This study was approved by the HNRCA Institutional Animal Care and
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11 Use Committee (IACUC) at Tufts University (protocol MM-45).
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14 15 16 **Sample preparation from animal study**

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18 At the end of the study (after 16 weeks), the mice sacrificed by asphyxiation with CO₂ and secondary
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20 euthanasia by cervical dislocation, what is consistent with the recommendations of the American
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22 Veterinary Medical Association. Mouse body weight, blood lipids and liver weight, histology and
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24 analysis of total lipids, cholesterol, triglycerides (TG), high density lipoprotein (HDL), low density
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26 lipoprotein (LDL) and glycogen have been previously reported (10). Samples of liver, kidney, brain,
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28 spleen, skeletal muscle, and adipose tissue (visceral, inguinal subcutaneous, interscapular subcutaneous)
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30 were frozen in liquid nitrogen and stored at -80°C for later preparation of extracts for Western blot
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32 analysis and cAMP measurements. Moreover, samples of these tissues were collected and stored in
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34 RNAlater solution (Qiagen) at -80°C for extraction of total RNA.
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40 **Quantitative RT-PCR**

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42 Tissues were homogenized with a polytron and total RNA was isolated using RNeasy Midi kit (Qiagen,
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44 Valencia, CA). cDNA was synthesized by TaqMan Reverse Transcription Reagents (Applied Biosystems,
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46 Foster City, CA). Gene expression of CD36, UCP1, UCP2, FABP4/aP2, and Sestrin 2 was measured by
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48 quantitative RT-PCR using primers and conditions of the TaqMan two-step PCR (Applied Biosystems,
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50 Foster City, CA). GAPDH was used as the endogenous control. The reactions were run in triplicate, and
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52 the data were analyzed using the $\Delta\Delta C_t$ method (31).
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58 **Preparation of tissue extracts and cAMP measurements**

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3 Frozen tissues samples (approx. 100 mg) were homogenized on ice in 500 μ L 2x Lysis buffer (diluted
4 from 10x Cell Lysis Buffer (Cell Signaling Technology, Beverly, MA)) containing protease inhibitors (1
5 tablet of protease inhibitor TABS (Roche)/10 mL, 0.5 mM IBMX (Sigma-Aldrich, St Louis, MI) and 100
6 μ M Ro20-1724 (Sigma-Aldrich)). Extracts were centrifuged 14,000 g for 15 min at 4°C to eliminate
7 debris or large fragments of DNA etc., and the protein concentration measured with BCA kit (Thermo
8 Scientific, Rockford, IL), aliquots of equal protein concentration prepared, and stored at -70°C. For
9 measuring cAMP levels, these aliquots were heated to 70°C for 10 min and centrifuged at 14,000 g at 4°C
10 for 10 min. Tissue cAMP levels in homogenized tissues (heart, liver, adipose tissues, intestine, skeletal
11 muscle, brain) were measured relative to cAMP standard using a Meso Scale cAMP assay system and
12 Sector Imager 2400 reader (Meso Scale Discovery, Gaithersburg, MD). The same extracts were used for
13 Western blotting.
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30 **cAMP measurements in cultured cells**

31 Cellular cAMP levels in response to Cur or THC were measured with the cAMP-Glo assay according to
32 the manufacturer's protocol (Promega, Madison, WI) using a GloMax®-Multi Microplate Multimode
33 Reader (Promega, Madison, WI).
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40 **Real-time cAMP measurements in cultured cells**

41 Real-time cellular cAMP levels in response to Cur and Cur metabolites in HEK293 cells were measured
42 using the cAMP Glo sensor assay system (Promega). For long term treatments (24 h), cells were
43 transfected with the cAMP reporter vector pGloSensorTM-22F (Promega, Madison, WI) for 3 h, then
44 treated with Cur analogues at the indicated concentrations for 24 h, upon which the medium was changed
45 to GloSensor reagent, pre-equilibrated at room temperature for 1 h, and the cAMP levels measured at
46 different time points thereafter according to the supplier's protocol using a GloMax®-Multi Microplate
47 Multimode Reader (Promega). For short term measurements (0-6 h), the cells were grown for 24 h after
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3 transfection and the treatment with Cur analogues occurred after changing medium to GloSensor reagent
4 and pre-equilibrating at room temperature for 1 h.
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9 10 **Plasmids, transfection and luciferase reporter assays**

11 The human CD36 promoter firefly luciferase reporter plasmids used were pCDextpro containing 4557 bp
12 of the human CD36 promoter (8), and pCDbasic (32, 33) containing only a short promoter. CD36
13 promoter deletions constructs were generated from pCDextpro (8) deleting parts by restriction digestion,
14 filling the ends with Klenow polymerase fragment when necessary and religation, resulting in plasmids
15 pCDΔEcoRI, pCDΔMluI/PvuII, pCDΔMluI/StuI, pCDΔStuI, and pCDΔMluI/MluNI. THP-1 monocytes
16 or HEK293 cells were transfected with Fugene (Promega) for 3h with the indicated reporter plasmids and
17 then treated with 0.1% ethanol (solvent control) or curcumin for an additional 21h. Extracts were
18 prepared, and promoter activities were measured using the Dual-Luciferase assay kit (Promega, Madison,
19 WI) with a GLOmax luminometer (Promega). The promoter-*Firefly* luciferase activities were normalized
20 to the thymidine kinase promoter-*Renilla* luciferase activities (pRL-TK), and the activities of the control
21 transfections were set to 100%.
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38 **Measurement of CREB, NRF2 and PPAR γ activity**

39 The activity of the transcription factor CREB was measured by transfecting luciferase reporter vectors for
40 CREB (pCREB-RE-luc, Clontech, Mountain View, CA), NRF2 (pARE-RE-luc (34)) or PPAR γ (pDR1-
41 Luc (35)). These plasmids were transfected into HEK293 or THP-1 cells together with the *Renilla* internal
42 control plasmid pRL-TK (Promega) for 3 h using Fugene (Promega) as transfection reagent. Extracts
43 were prepared, and promoter activities were measured as described above.
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53 **Western blotting**

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3 Cells (2×10^6 cells in 10 mL media per dish) were harvested and Western blots prepared as previously
4 described (8). For tissues, extracts were prepared as described above for cAMP measurements. The level
5 of CREB phosphorylation was determined using primary anti-phospho-CREB(Ser473), primary anti-
6 CREB antibody (all from Cell Signalling Technology), and horseradish peroxidase coupled donkey anti-
7 rabbit IgG secondary antibody (Amersham Biosciences, Piscataway, NJ). CD36 was measured using
8 monoclonal rabbit anti-mouse CD36 antibody, Western blots for UCP1 and UCP2 (supplementary Figure
9 1) were measured using polyclonal rabbit anti-mouse UCP1 and anti-human UCP2 antibodies (all from
10 Abcam, Cambridge, MA). Monoclonal mouse anti-human β -actin (1:10,000 diluted) (Sigma-Aldrich) was
11 used as primary control antibody for liver and cell culture extracts, and mouse monoclonal anti-GAPDH
12 (Abcam) for skeletal muscle extracts. Anti-mouse and anti-rabbit secondary antibodies coupled to
13 horseradish peroxidase (Santa Cruz, Santa Cruz, CA) were used for detection. Proteins were visualized
14 with an enzyme-linked chemiluminescence detection kit (Immun-Star™ HRP) according to the
15 manufacturer's instructions (Biorad, Hercules, CA). Chemiluminescence was monitored by exposure to
16 film (Kodak BioMax), and the signals were analyzed using a Fluorchem™ 8900 workstation and the
17 AlphaEaseFC software (AlphaInotech, San Leandro, CA).
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38 **Statistical analysis**

39 All values are expressed as the mean \pm standard errors (SEM) as inscribed in the figure legends. Student's
40 *t*-test was used to analyze the significant differences between two treatments. One-way ANOVA followed
41 by Tukey's post hoc test was used for multiple comparisons. $P < 0.05$ is considered to indicate a
42 significant difference. All data were analyzed using XLstat.
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51 **Results**

52 **Curcumin restores HFD-reduced levels of cAMP and increases CREB phosphorylation in the liver**

53 To investigate whether the previously observed regulatory effects of Cur on hypolipidemia (8, 14, 15)
54 possibly occur via activation of the cAMP/PKA/CREB pathway that plays an important role in lipid
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3 homeostasis, energy expenditure and thermogenesis, we measured the effect of Cur on cAMP levels in
4 the liver and several other tissues of *Ldlr*^{-/-} mice treated with LFD, or a HFD and a HFD supplemented
5 with three doses of Cur (500, 1,000 and 1,500 mg/kg) for 16 weeks. In the liver, a HFD significantly
6 decreased liver cAMP levels compared to LFD, and Cur increased it (**Figure 1A**). A similar but not
7 significant effect of HFD and Cur was observed in adipose tissues (36) collected from inguinal
8 subcutaneous and visceral fat depots (**Figure 1B**), but less in interscapular subcutaneous fat depots
9 (**Figure 1B**), in spleen, kidney, skeletal muscle and brain tissues (**Figure 1C**). In liver, where the Cur
10 effect on cAMP was strongest, Cur also increased the p-CREB/CREB ratio, suggesting that it may
11 contribute to the regulatory effects of Cur on gene expression mainly observed in this tissue (**Figure 1D**)
12 (37).
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27 **A high fat diet and curcumin increase CD36 gene expression in liver of *Ldlr*^{-/-} mice**

28 *Ldlr*^{-/-} mice lack the LDL receptor, the major route for lipid uptake by the liver, suggesting that HFD
29 induced steatohepatosis may result from the HFD increased expression of another receptor such as the
30 CD36/FAT scavenger receptor/fatty acids transporter, which at least in humans is up-regulated during
31 non-alcoholic fatty liver disease (NAFLD) (38). Based on this assumption, the level of CD36 mRNA and
32 protein was measured by quantitative PCR and Western blotting in the liver samples of the above mice.
33 HFD increased liver CD36 expression at mRNA and protein levels (**Figures 2A**), the stronger induction
34 of CD36 at the mRNA level when compared to the protein level may suggest some post-transcriptional
35 regulation such as alternative splicing, protein turnover (33, 39), or regulatory effects of microRNAs that
36 are regulated by curcumin (40). Supplementing the HFD with 500 mg/kg Cur slightly increased the
37 mRNA expression of CD36, whereas higher levels of Cur (1000 and 1,500 mg/kg diet) slightly reduced it
38 (**Figures 2A**). CD36 expression at the protein level was slightly increased by Cur (500 and 1000 mg/kg
39 diet) in liver, and again decreased at the highest Cur concentration (1,500 mg/kg diet) (**Figure 2B**). Much
40 weaker regulatory effects were observed in skeletal muscle, where HFD rather decreased CD36
41 expression, but Cur at low concentration (500 mg/kg diet) still increased it, whereas Cur at higher
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3 concentrations (1000 and 1,500 mg/kg diet) again decreased it (**Figure 2C**). Similar patterns of gene
4 expression was measured in liver for the fatty acid binding protein 4 (FABP4/aP2), uncoupling proteins 1
5 and 2 (UCP1/2), and sestrin 2 (SESN2), but they were weaker and mostly not significant (**supplemental**
6 **Figure 1**). Whereas in other tissues such as skeletal muscle and brain, no regulatory effects on these
7 genes were observed (**supplemental Figure 2**). Thus, when comparing the cAMP data (**Figure 1**) with
8 the CD36 gene expression data (**Figure 2**), it appears that in HFD-treated mice Cur can increase cAMP
9 levels and CD36 expression, but that in liver the induction of CD36 expression by Cur is lower since
10 HFD already robustly up-regulates CD36 expression by alternative pathways such as PPAR γ activation
11 (41, 42, 15) or by Ca²⁺ signaling in response to fatty acids sensing (43).
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25 **Curcumin increases cAMP and activates CREB in cultured cells**

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27 CD36 expression is regulated by several transcription factors, of which PPAR γ (44), SREBPs 1c and 2
28 (45), NRF2 (46-48) and CREB (49) have been shown to be responsive to Cur supplementation and
29 elevated lipids (reviewed in (11)). To assess which transcription factor(s) may be involved in the effects
30 observed with HFD and Cur treatments and whether a similar regulation also occur in human cells,
31 reporter vectors containing response elements for PPAR γ , NRF2 and CREB in front of the luciferase gene
32 were transfected into HEK293 cells, treated with Cur (5 and 10 μ M) for 24 h, and the transcriptional
33 activity measured. These cells were used as a model system since they are of epithelial origin, are
34 efficiently transfected and are able to take up Cur and metabolize it (50). Cur slightly activated PPAR γ
35 and NRF2, but the effects were strongest for CREB (**Figure 3A**), which might be activated by
36 cAMP/PKA (51, 52)). To investigate whether CREB could be involved in the up-regulation of the human
37 CD36 promoter in response to Cur, cells were treated with the bona fide adenylate cyclase (AC)
38 stimulator forskolin (10 μ M) which activates CREB by increasing the level of cAMP. Indeed, the CD36
39 promoter construct was stimulated by forskolin (**Figure 3B**) suggesting that CREB can be involved in the
40 up-regulation of CD36 expression induced by Cur.
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3 Cur is rapidly metabolized in cultured cells and some metabolites may have a higher activity (50).
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5 To evaluate whether metabolites of Cur were responsible for the effects on cAMP and CREB-RE activity,
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7 the major metabolites of Cur, such as tetrahydrocurcumin (THC), curcuminglucuronide (CurG),
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9 curcuminsulphate (CurS), demethoxycurcumin (DMCur), and bisdemethoxycurcumin (BDCur) were
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11 tested (53). Interestingly, with the exception of THC, these metabolites increased CREB-RE activity with
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13 similar efficiency as Cur within 24 h (**Figure 3C**), suggesting that Cur conversion to active metabolites
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15 may also contribute to its bioactivity. Differences measured between Cur and THC in activating cAMP
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17 and CREB phosphorylation could be due to their different ability to inhibit phosphodiesterases (PDE) or
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19 to stimulate adenylate cyclases (AC) activity (54, 55), as reported for resveratrol (28). At the molecular
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21 level, Cur and THC may have different affinity to receptors and enzymes and/or be converted to different
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23 metabolites.
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29 **A distant promoter fragment containing a consensus CREB-response element mediates the** 30 **stimulatory effects of Cur on CD36 expression** 31 32

33 The mechanisms of CD36 promoter regulation have been investigated in a number of studies and several
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35 regulatory elements (PPAR γ , NRF2, HIF1 α , SREBP1/2, LXR, PXR, C/EBP α/β) have been identified in
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37 the mouse gene (56, 47, 57, 58, 14). The organization of the human CD36 gene is complex (39) and the
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39 location of these regulatory elements in the promoter has not yet been resolved. To narrow down the
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41 regulatory region that is involved in the regulation of the human CD36 promoter, several deletions were
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43 introduced and the effect of Cur tested. Cur induced CD36 promoter activity with the full length construct
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45 (pCDextpro), as well as with the Δ MluI/MluNI construct (pCD Δ M/MI), but not with any of the other
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47 constructs, suggesting that the Cur responsive element is within a MluNI/EcoRI fragment of 480 bp
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49 (**Figure 4A**). Analysis of the sequence of this fragment by the TFSEARCH program revealed the
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51 presence of a bona fide consensus binding site for CREB (TGACCTCA) suggesting that Cur may act via
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53 this element (**Figure 4B**). Interestingly, deletion of the MluI/MluNI fragment (pCD Δ M/MI) also released
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3 the CD36 promoter from a inhibitory effect of basal promoter activity, and analysis of the MluI/MluNI
4 fragment revealed the presence of a bona fide NRF2 response element (ATGAATCAG) (not shown),
5 which can both stimulate or inhibit transcriptional activity depending whether positive (NRF2) or
6 negative (BACH1, MAF) regulatory factors are bound (59). Close to the CREB binding site, homologies
7 to consensus sites to other transcription factors were detected (HIF1 α , estrogen receptor (ER), SREBP-
8 1/2 (not shown)), altogether suggesting that the human promoter contains similar elements as the mouse
9 promoter but that they are clustered in a more distant region from the transcriptional start site. The
10 combined action of these elements may contribute to the regulatory effects observed of Cur on CD36
11 expression.
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25 **Real-Time assay of cAMP cellular concentration after treatment with various curcumin analogues**

26 Since the above cell culture experiments were all done with an incubation time of 24 h, differences
27 measured between Cur and THC on cAMP levels and CREB-RE activity could also be due to differences
28 in uptake and metabolism within this time-frame. In fact, we have recently shown that Cur is rapidly
29 taken up and still detectable 24 h after treatment of THP-1 cells, whereas THC is rapidly metabolized and
30 becomes un-detectable (50). A similar differential metabolism of Cur and THC was observed in HEK293
31 cells (**supplemental Figure 3**).
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40 To assess at which time point cAMP levels are increased leading to activation of CREB-RE, a
41 continuous real-time cAMP assay was used. In this assay, HEK293 cells were transfected with a cAMP
42 reporter vector pGloSensorTM-22F expressing a cAMP-luciferase reporter protein, which emits light upon
43 binding of cAMP and of the substrate Luciferin. Interestingly, upon short term treatment with Cur or
44 THC (10 μ M) for 0-6 h, THC had a slightly higher ability to increase cAMP levels in HEK293 cells, and
45 CurG was even higher whereas BDCur was lower (**Figure 5A**). A robust increase was observed after 2 h
46 with all compounds tested, and occurred at the concentration of at least 2.5 μ M (**Figure 5B**).
47 Interestingly, after 24 h, Cur was more effective in increasing cAMP levels when compared to THC
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3 (Figure 5C), which is similar to that of results obtained for CREB-RE activity reporter assay (Figure
4 3C), suggesting that differential uptake and metabolism are at the basis of CD36 promoter activity
5 differences seen with Cur and THC after 24 h. By comparing the effect of Cur with the one of known
6 controls, such as the phosphodiesterase (PDE) inhibitor 3-isobutyl-1-methylxanthine (IBMX) and the
7 adenylyl cyclase (AC) activators (adenosine, forskolin), it is concluded that the effects of Cur are slower
8 and weaker than adenosine and forskolin but similar in strength as IBMX (Figure 5D). An analogues
9 although weaker regulatory effect of Cur on cAMP levels and CREB-RE activity was observed in THP-1
10 monocytes (supplemental Figure 4).
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20 Discussion

21 The liver is an organ with central metabolic functions, including lipogenesis, gluconeogenesis, and
22 cholesterol synthesis as well as the primary site of metabolism for natural dietary components (60-62).
23 Macro and micronutrients such as fatty acids and Cur influence the metabolism of the liver and its ability
24 to maintain a normal plasma lipid homeostasis, with consequent lowering the risk of diseases in which
25 hyperlipidemia and lipid-mediated stress play important roles, such as inflammation, steatohepatosis,
26 diabetes, obesity, and atherosclerosis (reviewed in (9, 63, 11)). In fact, the liver has been reported to show
27 the strongest transcriptional response upon supplementation by Cur, whereas in other organs such as
28 intestine, kidney and spleen it was weaker or absent (37).
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42 In liver, we found increased CD36 expression in response to a HFD compared to a LFD, and Cur
43 (500 and 1000 mg/kg diet) slightly increased its expression at the protein level. Although the effects were
44 weak, they might be relevant since they may occur only in a subset of cells in tissues such as endothelial
45 or epithelial cells that are involved in the uptake of lipids. CD36, also named fatty acids transporter
46 (FAT), is an important regulator of plasma lipid levels (64), as demonstrated in several experimental
47 systems in which the level of expression of CD36 has been modified, e.g. by over-expressing CD36 or in
48 CD36 knockout mice. These experiments revealed that the influence of CD36 on plasma lipids depends
49 on the organ in which CD36 was induced or inhibited (65, 66), and that some organs/tissues are critically
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3 dependent on fatty acids influx by CD36 and for others too much fatty acid influx may not be desirable. It
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5 is important to note that patients with CD36 deficiency have increased plasma free fatty acid levels and
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7 increased atherosclerotic cardiovascular disease (67), which suggest that CD36 mediated signalling and
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9 lipid removal from the circulation is a protective mechanism. In contrast, since heart steady state
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11 energetic metabolism relies mostly on fatty acids, deficiency of CD36 can lead to hypertrophic
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13 cardiomyopathy and metabolic syndrome (68). In the liver, CD36 deletion reduces VLDL secretion and
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15 increases hepatic steatosis (69), suggesting that up-regulation of CD36 by Cur may have protective
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17 effects. The here observed increase of CD36 expression in the liver by HFD and Cur supplementation
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19 may particularly be relevant for postprandial uptake of excess fatty acids mediated by insulin-activated
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21 Akt/PPAR γ /CD36 expression. In the absence of insulin (e. g. diabetes) or during impaired insulin
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23 signalling (e. g. insulin resistance), a lower activation of CD36 expression may lead to insufficient
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25 removal of plasma lipid with consequent hyperlipidemia, and it remains to be investigated whether Cur
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27 can also increase CD36 in this situation (70).
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32 In skeletal muscle, we found that HFD down-regulated CD36 expression, which can be
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34 considered as protection of muscle against accumulation of lipids when lipids are abundant and not
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36 needed, e.g. in the absence of increased energy demand induced by exercise, but Cur supplementation still
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38 had an inducing effect on CD36 expression. Again, this may help in reducing hyperlipidemia, since
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40 muscle-specific over-expression of CD36 is associated with lower levels of body fat, serum free fatty
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42 acids and triacylglycerides and higher fasting glucose and insulin levels (71).
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46 Several transcription factor have been shown to be responsive to Cur, and some of them (e.g.
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48 PPAR γ , SREBP-1/2, NRF2, CREB) have a regulatory role for CD36 expression (reviewed in (11)). Using
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50 promoter deletion analysis, the regulatory effects of Cur were narrowed down to a 480 bp fragment
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52 containing a bona fide CREB response element along with a number of less conserved elements (HIF1 α ,
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54 NRF2, ER, SREBP), but the exact Cur-response element(s) within the human CD36 promoter remain to
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56 be determined by a detailed analysis by mutations, footprinting, and chromatin immunoprecipitation.
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3 Although Cur could increase PPAR γ activity in cell culture, activation of the human CD36 promoter by
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5 Cur was independent of PPAR γ and FOXO3a activation (8, 15), indicating involvement of other
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7 transcription factors. In cells, Cur stimulated NRF2, Hif1 α and more strongly CREB activity suggesting
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9 that these factors may play a role in up-regulating CD36 expression. Moreover, since the response of
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11 CD36 to HFD was different in liver and skeletal muscle, tissue specific regulatory mechanisms may be
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13 involved, such as HFD-induced liver CD36 expression as result of PPAR γ activation (41, 42, 15).
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17 At the molecular level, we find in liver and less in adipose tissue (visceral, inguinal
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19 subcutaneous) and spleen that HFD significantly decreases and Cur increases tissue cAMP levels,
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21 whereas no significant effect was observed in interscapular subcutaneous fat, brain, skeletal muscle and
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23 kidney. We recently reported that Cur is rapidly metabolized in cells (50), which may imply that the
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25 regulatory effects on cAMP may be caused not necessarily by Cur only but also by its metabolites. In fact,
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27 in HEK293 cells the increase of cAMP levels occurred within 4 h at which the Cur metabolite THC had
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29 the strongest effect, whereas after 24 h (the incubation time used for promoter assays), cAMP levels were
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31 highest with Cur rather than Cur metabolites such as THC, CurG, BDCur. Therefore, delayed uptake and
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33 slower metabolism of Cur when compared to THC may be responsible for its higher stimulatory effect on
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35 CD36 expression observed at 24 h. The level of cAMP influences the activities of protein kinase A (PKA)
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37 and of the transcription factor CREB. These proteins play important roles in lipid homeostasis, energy
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39 expenditure and thermogenesis by increasing lipolysis and fatty acid oxidation through stimulation of
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41 hormone sensitive lipase (HSL) and perilipin A (16, 72, 17). Thus, according to our data and as outlined
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43 in the proposed molecular model (**Figure 6**), an increase in cAMP levels induced by Cur could be at the
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45 basis of its hypolipidemic and anti-atherosclerotic effects and similar to other nutritional supplements
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47 such as forskolin or caffeine may also explain its effect on weight loss (20, 21, 29).
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52 Although it was reported that CREB expression was decreased in wild type mice after 2 weeks on
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54 a HFD (41), in our Ldlr^{-/-} mice treated for 16 weeks we find slightly elevated CREB phosphorylation in
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56 spite of decreased cAMP. CREB phosphorylation was stimulated by Cur probably as a result of
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3 increasing cAMP (51, 52)). In line with this notion, Cur activated cAMP and CREB activity in cultured
4 cells. CREB is mainly activated by phosphorylation by cAMP-activated protein kinase A (PKA) and
5 subsequent nuclear translocation (73), but alternative activation mechanisms may also be involved such as
6 the EPAC/PI3K/Akt pathway (74). It remains to be resolved whether Cur increases cAMP similar as
7 resveratrol (28) by inhibiting phosphodiesterases (PDE), or by stimulating adenylate cyclases (AC)
8 activity (54, 55). CREB is also activated by non-esterified fatty acids (NEFA) in vascular smooth muscle
9 cells (VSMC) most likely as a protective response to NEFA-mediated stress (75). Since CREB is down-
10 regulated in aortic VSMC in a high-fat diet induced model of vascular disease (73), Cur may normalize
11 CREB and thus be atheroprotective. Accordingly, increased cAMP decreases lipid content in adipocytes
12 and in atherosclerotic lesions (76).
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25 At this time we do not rule out that some of the effects of Cur on lipid homeostasis may also be
26 mediated by the CREB-related proteins such as cAMP response element binding protein hepatocyte
27 specific (CREBH) or cAMP response modulator (CREM), since they also play crucial roles in hepatic
28 lipogenesis, fatty acid oxidation, and lipid metabolism by controlling a number of genes involved in
29 triglycerides metabolism and influence plasma levels of free fatty acids, triglycerides and cholesterol (77,
30 46, 78). CREBH is activated by metabolic stress induced by saturated fatty acids, insulin signals, or an
31 atherogenic high-fat diet in the liver (79), and Cur reduced endoplasmic reticulum (ER) stress by
32 inhibiting CREBH/CREB3L3 by stimulating the LKB1/AMPK/SMILE/PGC1 α (51). Moreover, an
33 induction of cAMP and CREBH by Cur may not only contribute to its hypolipidemic but also its
34 hypoglycemic effects, e.g. by improving glucose metabolism and insulin resistance (80, 27, 81).
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47 A Cur-mediated increase of lipids uptake in liver in the absence of lipid accumulation may
48 require increased lipid excretion in bile (e.g. of cholesterol metabolites) (reviewed in (11)) or increased
49 channelling into anabolic pathways (e.g. protein and phospholipid synthesis), which is reflected in part by
50 the observed increase in liver size and preservation of glycogen content (10). Alternatively, Cur may
51 stimulate energy dissipation, e.g. by direct uncoupling of mitochondrial oxidative phosphorylation (82),
52 or by up-regulation of proteins involved in catabolic metabolism and metabolic inefficiency such as
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3 UCP1 (83) and AMPK/UCP2 (84-86). In fact, UCP1 is regulated by FOXO1 in liver (87) and UCP2
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5 expression is induced by obesity (88) and Cur (89).
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8 Taken together, our *in vivo* data suggest that Cur exerts regulatory effects on cAMP cellular
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10 concentrations predominantly in the liver. At this time we can only correlate the elevated cAMP levels
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12 with the *in vivo* Cur-induced hypolipidemic effects, although our *in vitro* experiments suggest a direct
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14 cause-effect relationship between Cur, cAMP, and the regulation of genes involved in lipid and energy
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16 homeostasis such as CD36, UCP1/2 and aP2. Since the liver centrally participates in the regulation of
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18 plasma lipid, cholesterol and glucose levels (60-62), it remains to be evaluated to what degree the
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20 observed effects of Cur in other organs are in fact secondary effects of regulatory events occurring in
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22 liver.
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24 25 26 27 **Acknowledgments**

28
29 *This study was supported by a grant of the US Department of Agriculture, under Agreement No. 58-1950-*
30
31 *0-014. EC was recipient of an IUBMB Wood-Whelan Fellowship; KN was recipient of a sabbatical*
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33 *fellowship from Tohoku University. We thank Stephanie Marco for her assistance in the preparation of*
34
35 *this manuscript. Any opinions, findings, conclusions, or recommendations expressed in this publication*
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37 *are those of the author(s) and do not necessarily reflect the view of the U.S. Department of Agriculture.*
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42 *JMZ, RR, AA, MM designed research, analyzed data, and wrote paper. JMZ, STH, KN, EC conducted*
43
44 *research, LV provided essential reagents. All authors read and approved the final manuscript. None of*
45
46 *the authors has declared any conflict of interest.*
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48 49 50 51 **Figures**

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53 **Fig 1. Quantification of cAMP levels in liver and adipose tissues.** (A) Livers from LDLr^{-/-} mice fed a
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55 low or high fat diet (LFD or HFD, respectively) supplemented for 16 weeks with various concentrations
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57 of low, medium, and high Cur (500, 1000, 1500 mg/kg diet, respectively) were homogenized and cAMP
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3 levels measured by Mesoscale cAMP assay ($n = 22$, \pm SEM, $*p < 0.05$, relative to control fed a LFD set to
4 100%). (B) Similar, but less pronounced results were obtained with adipose tissues (inguinal
5 subcutaneous, visceral and less with interscapular subcutaneous fat) ($n = 8$, \pm SEM), as well as with (C)
6 spleen ($n=8$, \pm SEM), but less with skeletal muscle, kidney, and brain ($n=8$, \pm SEM). (D) Regulation of
7 CREB phosphorylation by HFD and Cur as assessed Western blots of liver extracts. The level of
8 phospho-CREB was calculated relative to unphosphorylated CREB and plotted ($n=12$, \pm SEM, $*p < 0.05$,
9 with control (LFD) set to 100%).
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20 **Fig 2. Regulation of CD36 expression by high fat diet and Cur.** Quantitative RT-PCR with cDNA, or
21 western blots of liver extracts from *Ldlr*^{-/-} mice that were fed a LFD, a HFD and a HFD with a low,
22 medium and high dose of Cur (500, 1000, 1500 mg/kg diet, respectively) for 16 weeks. Expression of
23 liver CD36 mRNA (A), or CD36 protein (B) (expression of mRNA, $n=4$, \pm SEM, $*p < 0.05$ relative to
24 LFD, with control (LFD) set to 100%; expression of protein, $n=12$, \pm SEM, $*p < 0.05$ relative to LFD, with
25 control (LFD) set to 100%). Expression of skeletal muscle CD36 protein (C) ($n=8$, \pm SEM, $*p < 0.05$
26 relative to LFD, with control (LFD) set to 100%).
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38 **Fig 3. Cur activates PPAR γ , NRF2 and CREB activity in HEK293 cells.** (A) Treatment with Cur (5
39 and 10 μ M) for 24 h increases PPAR γ -RE, NRF2-RE and CREB-RE, as assessed by transfecting response
40 element luciferase reporter constructs for these transcription factors into HEK293 cells. (B) The human
41 CD36 promoter is controlled by cAMP/CREB. HEK293 cells were transfected with a human CD36
42 promoter luciferase reporter constructs and treated with forskolin (10 μ M) for 24 h ($n=4$, \pm SEM, $p < 0.05$,
43 with untreated control (c) set to 100%). (C) Differential effects of Cur metabolites on CREB-RE
44 luciferase activity. HEK293 cells treated with Cur, THC, CurG, DCur, BDCur, or CurS (all 10 μ M) for 24
45 h and luciferase activity measured ($n=4$, \pm SEM, $*p < 0.05$, with untreated control (c) set to 100%, $\#p$
46 < 0.05 relative to Cur).
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6 **Fig 4. A distant site of the human CD36 promoter containing a CREB consensus site is responsive**
7 **to Cur.** (A) Treatment with Cur (10 μ M) for 24 h increases the full length (pCDextpro) and the
8 Δ MluI/MluNI CD36 promoter-luciferase reporter construct (pCDDM/MI), but not any of the other
9 constructs, as assessed by transfection of these constructs into HEK293 cells for 24 h (n=6, \pm SEM, *p
10 <0.05, with untreated pCDextpro control (c) set to 100%; #p <0.05, compared to transfected plasmid in
11 the absence of Cur). M: MluI, MI: MluNI, E: EcoRI, P: PvuII, S: StuI. (B) Sequence of the 480 bp Cur-
12 responsive region and identification of a bona fide consensus regulatory element for CREB.
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23 **Fig 5. Regulation of cAMP levels by Cur metabolites.** HEK293 cells were transfected with a cAMP
24 reporter vector pGloSensorTM-22F expressing a cAMP-luciferase reporter protein, which emits light upon
25 binding cAMP and then treated with Cur, THC, CurG or BDCur. (A) Time dependence of cAMP
26 accumulation after short term treatment (0 – 6 h) with Cur or Cur metabolites (all 10 μ M). (B)
27 Concentration dependence of cAMP accumulation after treatment with Cur or Cur metabolites (0.625 –
28 20 μ M) for 2 h. (C) Long term treatment (24 h) with Cur or Cur metabolites (all 10 μ M) (n=2, \pm SEM, *p
29 <0.05, relative to untreated control; #p <0.05, relative to treatment with Cur). (D) Accumulation of cAMP
30 after treatment for 0 – 6 h with the adenylate cyclase (AC) activator forskolin (10 μ M) or 2'-5'-
31 dideoxyadenosine (250 μ M), or the phosphodiesterase inhibitor IBMX (25 μ M).
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45 **Fig 6. Scheme of proposed molecular mechanisms involved in hypolipidemic effects of Cur.** Cur
46 increases cAMP in liver, either by increasing adenylate cyclase (AC) activity or inhibiting
47 phosphodiesterase (PE) activity. Increased levels of cAMP activate PKA/CREB which induces lipid
48 transport proteins (CD36, aP2), and genes involved in lipid metabolism (e.g. HSL, CPT-1) and energy
49 homeostasis (e.g. UCP1/2) leading overall to a hypolipidemic effect. Whether Cur also regulates lipid
50 export from adipose tissue by regulating HSL, aP2 or perilipin 2 remains to be shown (grey letters).
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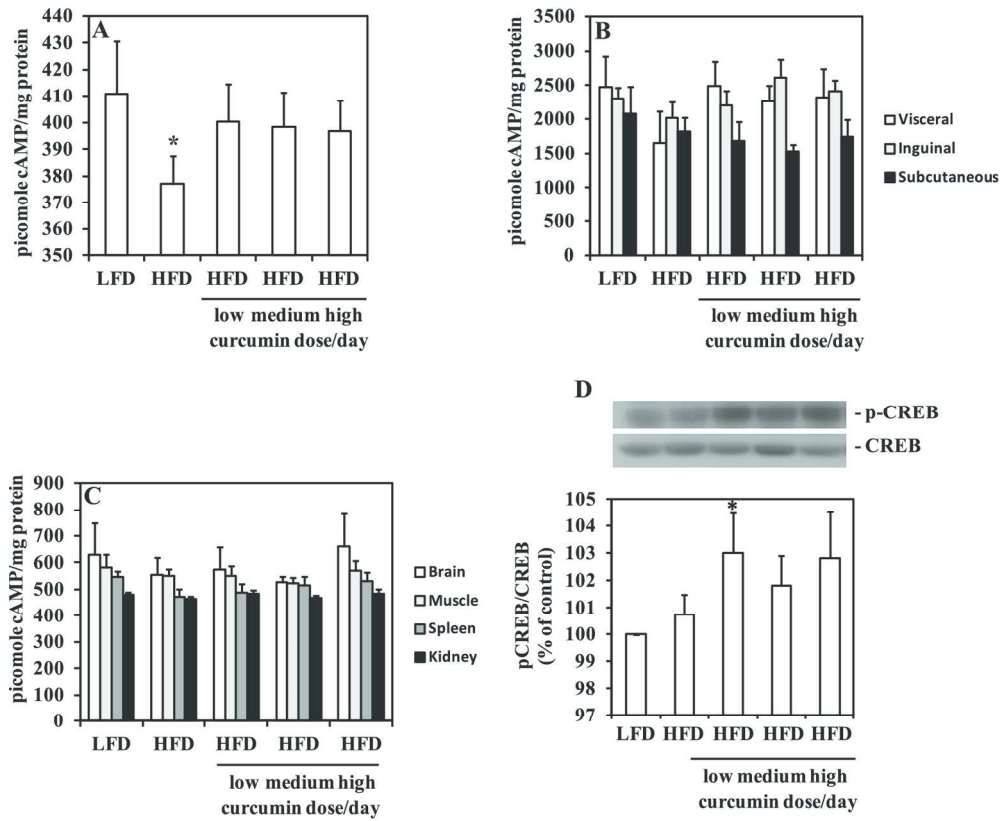


Figure 1
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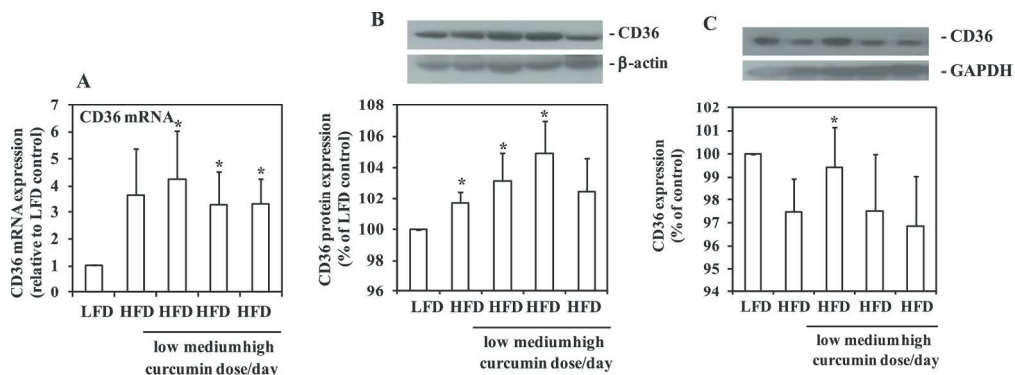


Figure 2
202x74mm (300 x 300 DPI)

Peer Review

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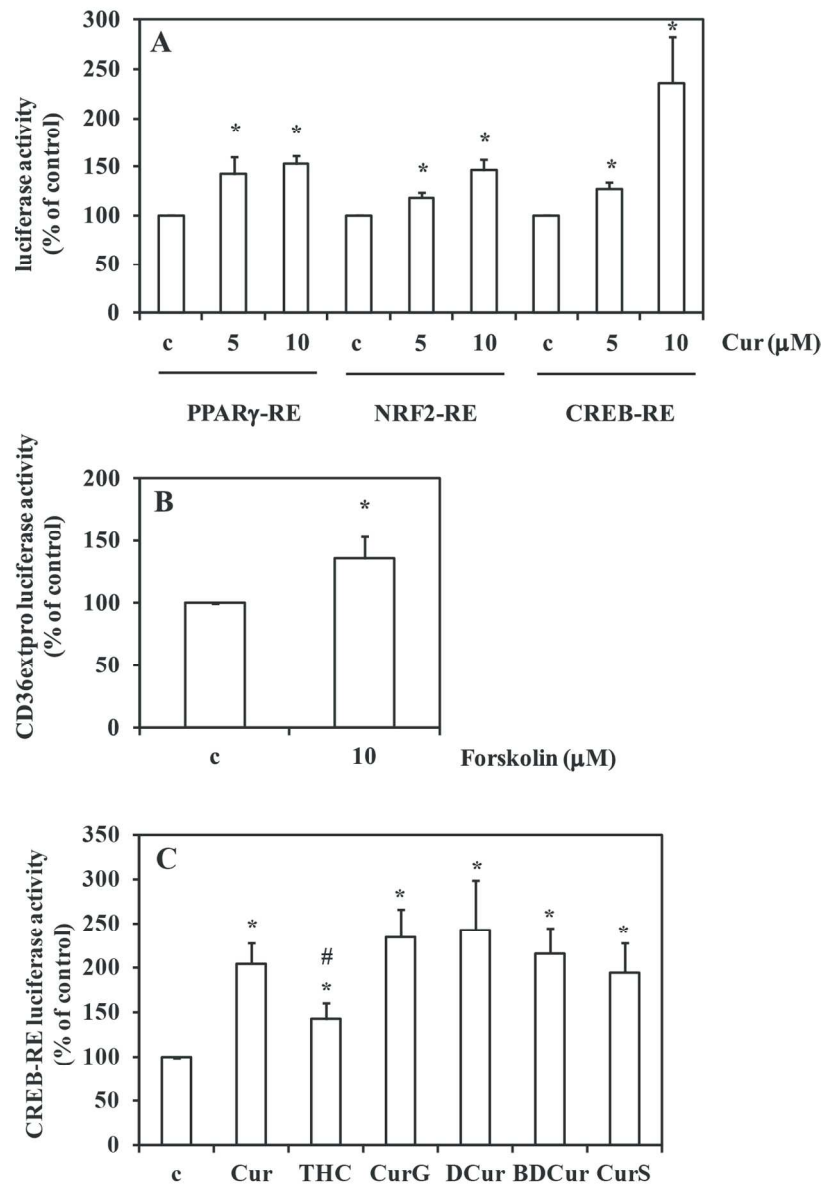
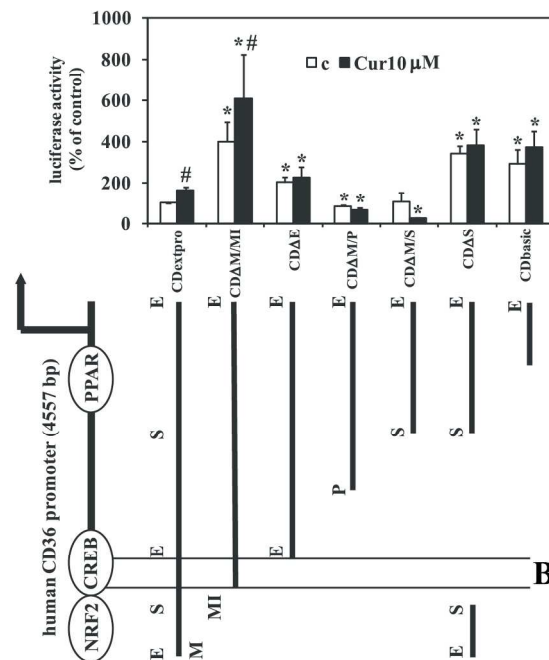


Figure 3
112x167mm (300 x 300 DPI)

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TATAGTGGCACAATCTTAGCTCACTGCAAACCTCCGCCTCCCAGGTTCAAGCGATTTTCTGTCTCAGCC

TCCTGAGTAGTTGGGATTACAGGCATGCACCACCACCTGGCTAATTTTTGTATTTTGTAGTAGAGGA

CREB

GGTTTCACCATGTTGGTCAAGCTACTCTCGAACTCCTGACCTCAAGTGATCCGCCACCTCAGCCTCCA

AAGTGTGGGATTACAGGTGTGAGCCACCAAGCCAGCCACAATTATTTGTTAAACAAAACTATGTCAG

GCTCTGTGCTAAATCCTTCATATTATCACTTTTAATCTTGACAGCAATCTGTGAGGGAGATATTTCTAC

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EcoRI

165x231mm (300 x 300 DPI)

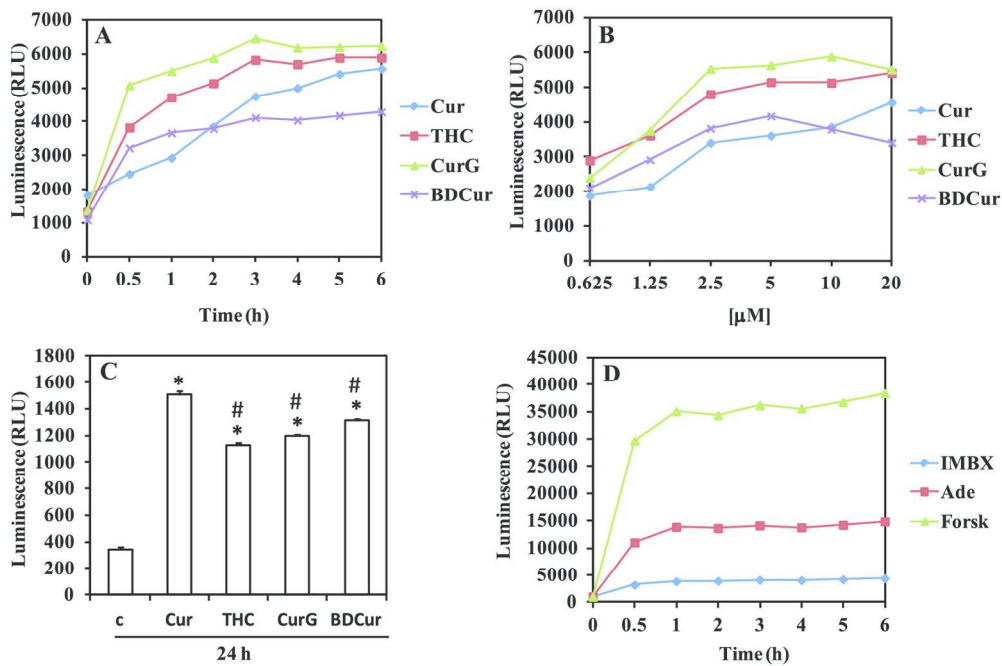


Figure 5
171x113mm (300 x 300 DPI)

Review

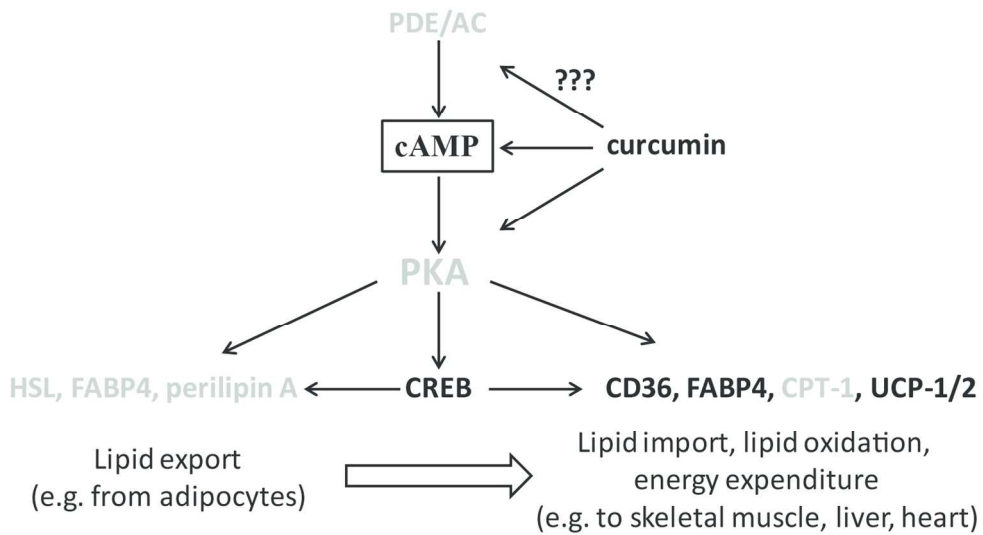


Figure 6
137x72mm (300 x 300 DPI)