Research Communication

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

Jean-Marc Zingg[1]*
Syeda T. Hasan[1]
Kiyotaka Nakagawa[1]
Elisa Canepa[2]
Roberta Ricciarelli[2]
Luis Villacorta[3]
Angelo Azzi[1]
Mohsen Meydani[1]

[1]*Vascular Biology Laboratory, JM USDA-Human Nutrition Research Center on Aging, Tufts University, Boston, MA 02111, USA*

[2]*Department of Experimental Medicine, Section of General Pathology, University of Genoa, Genoa, Italy*

[3]*Cardiovascular Center, Department of Internal Medicine, University of Michigan Medical Center, Ann Arbor, MI, USA*

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 this 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 an 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. © 2016 BioFactors, 43(1):42–53, 2017

Keywords: *CREB; NRF2; PPARγ; CD36; curcumin; cAMP signaling; gene expression; fat accumulation; fat metabolism; obesity; atherosclerosis; inflammation*

Abbreviations: *cAMP, cyclic adenosine monophosphate; Cur, curcumin; HFD, high-fat diet; Ldlr, low-density lipoprotein receptor; LDL, low-density lipoprotein*

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**Address for correspondence: Jean-Marc Zingg; Vascular Biology Laboratory, Office 621, JM USDA-Human Nutr. Res. Ctr. On Aging, Tufts University, 711 Washington St., Boston, MA 02111, USA. E-mail: Jean-marc.Zingg@tufts.edu*

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1. 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 refs. 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 ref. 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 [10,13]. 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 downregulates CD36 and aP2 with consequent lower lipid accumulation in these cells [8].

The plasma lipid level is predominately influenced by the interplay of liver, skeletal muscle, and adipose tissues. In these tissues, the cAMP/PKA/CREB signaling pathway plays important roles in lipid homeostasis by increasing PKA-mediated lipolysis and fatty acid oxidation [16,17]. In the liver, cAMP/PKA regulates lipid homeostasis by modulating energy expenditure and thermogenesis, by decreasing liver VLDL secretion and increasing lipolysis and fatty acid oxidation [18–21]. In skeletal/cardiac muscle, cAMP stimulates CD36-mediated lipid uptake, β-oxidation, and mitochondrial biogenesis [22,23], and protects against muscle atrophy by inhibiting the ubiquitin–proteasome system [24]. In skeletal muscle, lipid uptake is stimulated by CD36 membrane exposition and controlled by FOXO1 by yet unclear molecular mechanisms [25–27]. In adipocytes as well as in atherosclerotic lesions, increased cAMP decreases lipid content [18]. Thus, as described recently for the polyphenol resveratrol [28], upregulation of tissue cAMP levels by Cur could be at the basis of its hypolipidemic and anti-atherosclerotic effects and similar to other nutritional supplements such as forskolin or caffeine, it may also explain its effect on weight loss [6,21,29].

In this study, using cell culture and *in vivo* animal models, we have investigated whether the induction of cAMP levels in different tissues and subsequent stimulation of lipid transporters and catabolic genes by Cur could be at the basis of its hypolipidemic effects. As an *in vivo* experimental model, we used the $Ldlr^{-/-}$ mouse which is a good animal model of high-fat-induced accelerated hyperlipidemia and atherosclerosis, since when fed a high-fat Western style diet, these mice show a high concentration of circulating cholesterol and develop lipid-mediated stress (accumulation of lipids, inflammation, increased oxidative stress) at 8 weeks [30]. Our results suggest that a regulatory role of Cur on cAMP-mediated signaling and gene expression may contribute to its effects on prevention of atherosclerosis and inflammation.

2. Materials and Methods

2.1. Materials

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

2.2. Cell Culture and Treatments

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

2.3. Animal Feeding Protocol

$Ldlr^{-/-}$ C57BL/6 male mice (Jackson Laboratory, Bar Harbor, ME) were individually housed at the Jean Mayer Human Nutrition Research Center on Aging (HNRCA) Comparative Biology Unit at Tufts University. The mice were kept on a 12 h light/dark cycle and had free access to food and water. After acclimation for 1 week, 8-week-old mice were randomly assigned to five groups of 24 mice each. One group of mice was fed an AIN-93M diet (Harlan Teklad, TF.08485) for 16 weeks and served as "low-fat diet (LFD) negative controls." The other 4 groups of mice were fed a Western style high-fat/cholesterol AIN-93M diet (HFD) (Harlan Teklad TD.09346) formulated to contain 0.157% cholesterol and 21% fat by weight for 4 months. AIN-93 mineral and vitamin mixes were used in the formulation of diets. From the HFD-fed mice, one group of mice was designated as positive HFD control, and the diets of the other three groups were either supplemented with a low (500 mg/kg), medium (1000 mg/kg), and high (1500 mg/kg)

dose of Cur for 16 weeks. This study was approved by the HNRCA Institutional Animal Care and Use Committee (IACUC) at Tufts University (protocol MM-45).

2.4. Sample Preparation from Animal Study

At the end of the study (after 16 weeks), the mice were sacrificed by asphyxiation with CO_2 and secondary euthanasia by cervical dislocation, what is consistent with the recommendations of the American Veterinary Medical Association. Mouse body weight, blood lipids and liver weight, histology and analysis of total lipids, cholesterol, triglycerides (TG), high-density lipoprotein (HDL), low-density lipoprotein (LDL), and glycogen have been previously reported [10]. Samples of liver, kidney, brain, spleen, skeletal muscle, and adipose tissue (visceral, inguinal subcutaneous, interscapular subcutaneous) were frozen in liquid nitrogen and stored at −80°C for later preparation of extracts for Western blot analysis and cAMP measurements. Moreover, samples of these tissues were collected and stored in RNA*later* solution (Qiagen) at −80°C for extraction of total RNA.

2.5. Quantitative RT-PCR

Tissues were homogenized with a polytron and total RNA was isolated using RNeasy Midi kit (Qiagen, Valencia, CA). cDNA was synthesized by TaqMan Reverse Transcription Reagents (Applied Biosystems, Foster City, CA). Gene expression of CD36, UCP1, UCP2, FABP4/aP2, and Sestrin 2 was measured by quantitative RT-PCR using primers and conditions of the TaqMan two-step PCR (Applied Biosystems, Foster City, CA). GAPDH was used as the endogenous control. The reactions were run in triplicate, and the data were analyzed using the ΔΔCt method [31].

2.6. Preparation of Tissue Extracts and cAMP Measurements

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

2.7. cAMP Measurements in Cultured Cells

Cellular cAMP levels in response to Cur or THC were measured with the cAMP-Glo assay according to the manufacturer's protocol (Promega, Madison, WI) using a GloMax®-Multi Microplate Multimode Reader (Promega, Madison, WI).

2.8. Real-Time cAMP Measurements in Cultured Cells

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

2.9. Plasmids, Transfection, and Luciferase Reporter Assays

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

2.10. Measurement of CREB, NRF2, and PPARγ Activity

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

2.11. Western Blotting

Cells (2×10^6 cells in 10 mL media per dish) were harvested and Western blots prepared as previously described [8]. For tissues, extracts were prepared as described above for cAMP measurements. The level of CREB phosphorylation was determined using primary anti-phospho-CREB (Ser473), primary

anti-CREB antibody (all from Cell Signaling Technology), and horseradish peroxidase coupled donkey antirabbit IgG secondary antibody (Amersham Biosciences, Piscataway, NJ). CD36 was measured using monoclonal rabbit antimouse CD36 antibody, Western blots for UCP1 and UCP2 (Supporting Information, Fig. 1) were measured using polyclonal rabbit antimouse UCP1 and antihuman UCP2 antibodies (all from Abcam, Cambridge, MA). Monoclonal mouse antihuman β-actin (1:10,000 diluted) (Sigma-Aldrich) was used as primary control antibody for liver and cell culture extracts, and mouse monoclonal anti-GAPDH (Abcam) for skeletal muscle extracts. Antimouse and antirabbit secondary antibodies coupled to horseradish peroxidase (Santa Cruz, Santa Cruz, CA) were used for detection. Proteins were visualized with an enzyme-linked chemiluminescence detection kit (Immun-Star™ HRP) according to the manufacturer's instructions (Biorad, Hercules, CA). Chemiluminescence was monitored by exposure to film (Kodak BioMax), and the signals were analyzed using a Fluorchem™ 8900 workstation and the AlphaEaseFC software (AlphaInotech, San Leandro, CA).

2.12. Statistical Analysis

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

3. RESULTS

3.1. Curcumin Restores HFD-Reduced Levels of cAMP and Increases CREB Phosphorylation in the Liver

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

3.2. A High-Fat Diet and Curcumin Increase CD36 Gene Expression in Liver of $Ldlr^{-/-}$ Mice

$Ldlr^{-/-}$ mice lack the LDL receptor, the major route for lipid uptake by the liver, suggesting that HFD-induced steatohepatosis may result from the HFD increased expression of another receptor such as the CD36/FAT scavenger receptor/fatty acids transporter, which at least in humans is upregulated during nonalcoholic fatty liver disease (NAFLD) [38]. Based on this assumption, the level of CD36 mRNA and protein was measured by quantitative PCR and Western blotting in the liver samples of the above mice. HFD increased liver CD36 expression at mRNA and protein levels (Fig. 2A), the stronger induction of CD36 at the mRNA level when compared to the protein level may suggest some post-transcriptional regulation such as alternative splicing, protein turnover [33,39], or regulatory effects of microRNAs that are regulated by curcumin [40]. Supplementing the HFD with 500 mg/kg Cur slightly increased the mRNA expression of CD36, whereas higher levels of Cur (1,000 and 1,500 mg/kg diet) slightly reduced it (Fig. 2A). CD36 expression at the protein level was slightly increased by Cur (500 and 1,000 mg/kg diet) in liver, and again decreased at the highest Cur concentration (1,500 mg/kg diet) (Fig. 2B). Much weaker regulatory effects were observed in skeletal muscle, where HFD rather decreased CD36 expression, but Cur at low concentration (500 mg/kg diet) still increased it, whereas Cur at higher concentrations (1,000 and 1,500 mg/kg diet) again decreased it (Fig. 2C). Similar patterns of gene expression was measured in liver for the fatty acid binding protein 4 (FABP4/aP2), uncoupling proteins 1 and 2 (UCP1/2), and sestrin 2 (SESN2), but they were weaker and mostly not significant (Supporting Information, Fig. 1). Whereas in other tissues such as skeletal muscle and brain, no regulatory effects on these genes were observed (Supporting Information, Fig. 2). Thus, when comparing the cAMP data (Fig. 1) with the CD36 gene expression data (Fig. 2), it appears that in HFD-treated mice Cur can increase cAMP levels and CD36 expression, but that in liver the induction of CD36 expression by Cur is lower since HFD already robustly upregulates CD36 expression by alternative pathways such as PPARγ activation [15,41,42] or by Ca^{2+} signaling in response to fatty acids sensing [43].

3.3. Curcumin Increases cAMP and Activates CREB in Cultured Cells

CD36 expression is regulated by several transcription factors, of which PPARγ [44], SREBPs 1c and 2 [45], NRF2 [46–48], and CREB [49] have been shown to be responsive to Cur supplementation and elevated lipids (reviewed in ref. 11). To assess which transcription factor(s) may be involved in the effects observed with HFD and Cur treatments and whether a similar regulation also occur in human cells, reporter vectors containing response elements for PPARγ, NRF2, and CREB in front of the luciferase gene were transfected into HEK293 cells, treated with Cur (5 and 10 μM) for 24 h, and the transcriptional activity measured. These cells were used as a model system since they are of epithelial origin, are efficiently transfected and are able to take up Cur and metabolize it [50]. Cur slightly activated PPARγ and NRF2, but the effects were strongest for CREB (Fig. 3A), which might be activated by cAMP/PKA [51,52]). To investigate whether CREB could be

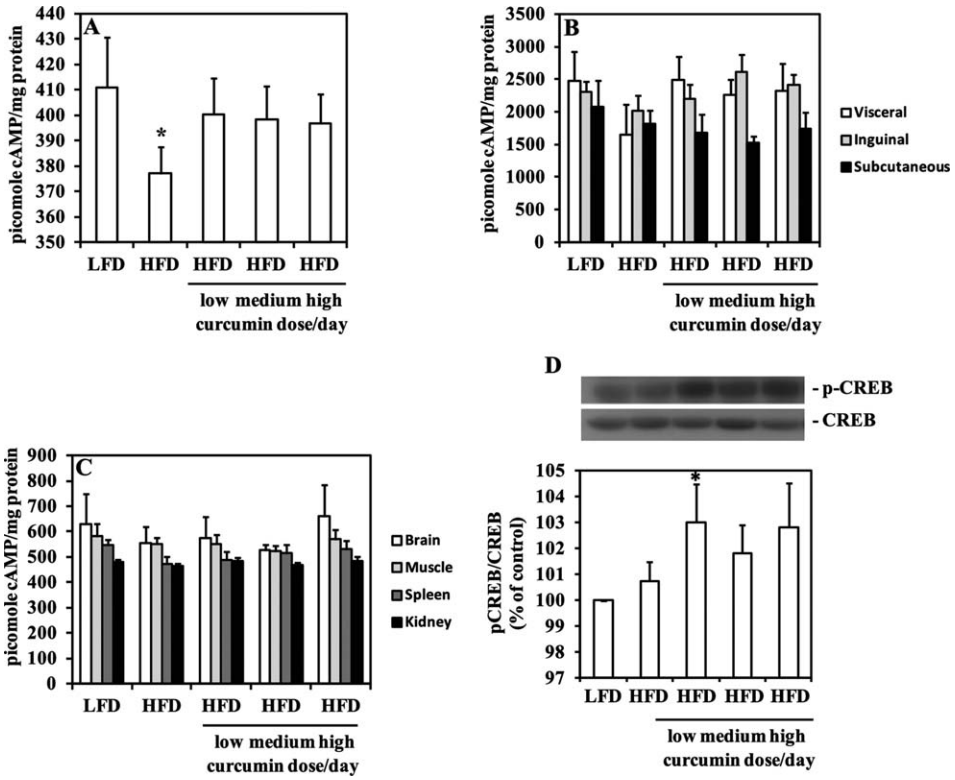

FIG 1

*Quantification of cAMP levels in liver and adipose tissues. (**A**) Livers from Ldlr$^{-/-}$ mice fed a low- or high-fat diet (LFD or HFD, respectively) supplemented for 16 weeks with various concentrations of low, medium, and high Cur (500, 1000, and 1500 mg/kg diet, respectively) were homogenized and cAMP levels measured by Mesoscale cAMP assay (*n* = 22, ±SEM, *P < 0.05, relative to control fed an LFD set to 100%). (**B**) Similar, but less pronounced results were obtained with adipose tissues (inguinal subcutaneous, visceral and less with interscapular subcutaneous fat) (*n* = 8, ±SEM), as well as with (**C**) spleen (*n* = 8, ±SEM), but less with skeletal muscle, kidney, and brain (*n* = 8, ±SEM). (**D**) Regulation of CREB phosphorylation by HFD and Cur as assessed Western blots of liver extracts. The level of phospho-CREB was calculated relative to unphosporylated CREB and plotted (*n* = 12, ±SEM, *P < 0.05, with control (LFD) set to 100%).*

involved in the upregulation of the human CD36 promoter in response to Cur, cells were treated with the bona fide adenylate cyclase (AC) stimulator forskolin (10 μM) which activates CREB by increasing the level of cAMP. Indeed, the CD36 promoter construct was stimulated by forskolin (Fig. 3B) suggesting that CREB can be involved in the upregulation of CD36 expression induced by Cur.

Cur is rapidly metabolized in cultured cells and some metabolites may have a higher activity [50]. To evaluate whether metabolites of Cur were responsible for the effects on cAMP and CREB-RE activity, the major metabolites of Cur, such as tetrahydrocurcumin (THC), curcuminglucuronide (CurG), curcuminsulphate (CurS), demethoxycurcumin (DMCur), and bisdemethoxycurcumin (BDCur) were tested [53]. Interestingly, with the exception of THC, these metabolites increased CREB-RE activity with similar efficiency as Cur within 24 h (Fig. 3C), suggesting that Cur conversion to active metabolites may also contribute to its bioactivity. Differences measured between Cur and THC in activating cAMP and CREB phosphorylation could be due to their different ability to inhibit phosphodiesterases (PDE) or to stimulate adenylate cyclases (AC) activity [54,55], as reported for resveratrol [28]. At the molecular level, Cur and THC may have different affinity to receptors and enzymes and/or be converted to different metabolites.

3.4. A Distant Promoter Fragment Containing a Consensus CREB-Response Element Mediates the Stimulatory Effects of Cur on CD36 Expression

The mechanisms of CD36 promoter regulation have been investigated in a number of studies and several regulatory elements (PPARγ, NRF2, HIF1α, SREBP1/2, LXR, PXR, C/EBPα/β) have been identified in the mouse gene [14,47,56–58]. The organization of the human CD36 gene is complex [39] and the location of these regulatory elements in the promoter has not yet been resolved. To narrow down the regulatory region that is involved in the regulation of the human CD36 promoter, several deletions were introduced and the effect of Cur tested. Cur induced CD36 promoter activity with the full-length construct (pCDextpro), as well as with the ΔMluI/MluNI construct (pCDΔM/MI), but not with any of the other constructs, suggesting that the Cur responsive element is within a MluNI/EcoRI fragment of 480 bp (Fig. 4A). Analysis of the sequence of this fragment by the TFSEARCH program revealed the presence of

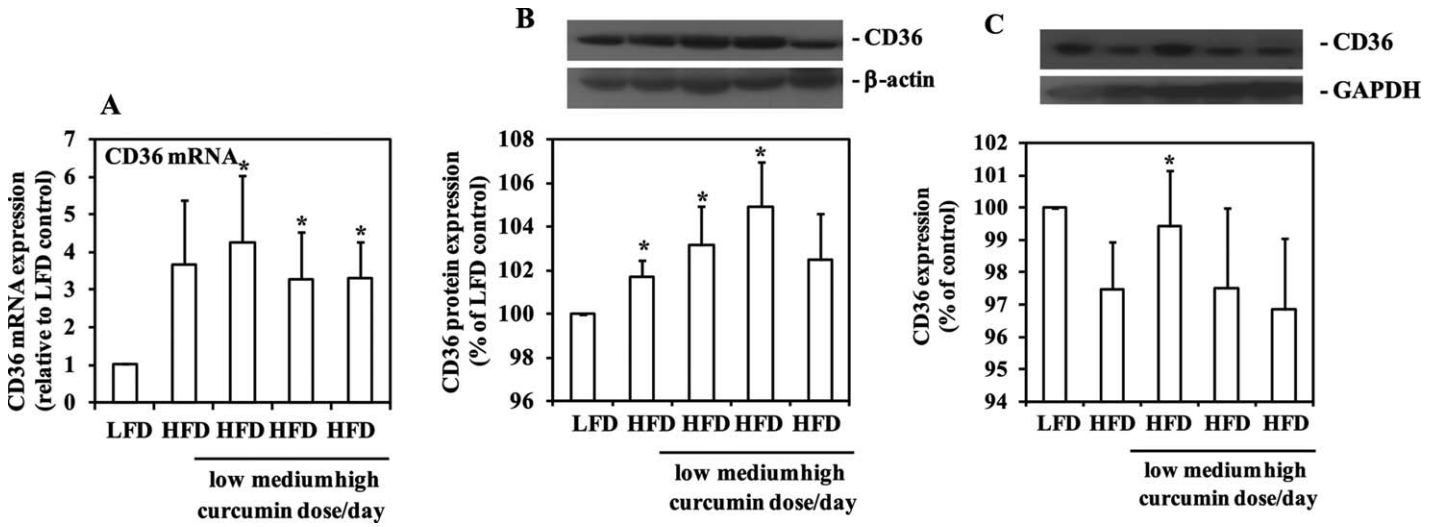

FIG 2

*Regulation of CD36 expression by high-fat diet (HFD) and Cur. Quantitative RT-PCR with cDNA, or western blots of liver extracts from Ldlr$^{-/-}$ mice that were fed an LFD, an HFD, and an HFD with a low, medium, and high dose of Cur (500, 1000, and 1500 mg/kg diet, respectively) for 16 weeks. Expression of liver CD36 mRNA (**A**) or CD36 protein (**B**) (expression of mRNA, n = 4, ±SEM, *P < 0.05 relative to LFD, with control (LFD) set to 100%; expression of protein, n = 12, ±SEM, *P < 0.05 relative to LFD, with control (LFD) set to 100%). Expression of skeletal muscle CD36 protein (**C**) (n = 8, ±SEM, *P < 0.05 relative to LFD, with control (LFD) set to 100%).*

a bona fide consensus binding site for CREB (TGACCTCA) suggesting that Cur may act via this element (Fig. 4B). Interestingly, deletion of the MluI/MluNI fragment (pCDΔM/MI) also released the CD36 promoter from an inhibitory effect of basal promoter activity, and analysis of the MluI/MluNI fragment revealed the presence of a bona fide NRF2 response element (ATGAATCAG) (not shown), which can both stimulate or inhibit transcriptional activity depending whether positive (NRF2) or negative (BACH1, MAF) regulatory factors are bound [59]. Close to the CREB binding site, homologies to consensus sites to other transcription factors were detected (HIF1α, estrogen receptor (ER), SREBP-1/2 (not shown)), altogether suggesting that the human promoter contains similar elements as the mouse promoter but that they are clustered in a more distant region from the transcriptional start site. The combined action of these elements may contribute to the regulatory effects observed of Cur on CD36 expression.

3.5. Real-Time Assay of cAMP Cellular Concentration after Treatment with Various Curcumin Analogs

Since the above cell culture experiments were all done with an incubation time of 24 h, differences measured between Cur and THC on cAMP levels and CREB-RE activity could also be due to differences in uptake and metabolism within this timeframe. In fact, we have recently shown that Cur is rapidly taken up and still detectable 24 h after treatment of THP-1 cells, whereas THC is rapidly metabolized and becomes undetectable [50]. A similar differential metabolism of Cur and THC was observed in HEK293 cells (Supporting Information, Fig. 3).

To assess at which time point, cAMP levels are increased leading to activation of CREB-RE, a continuous real-time cAMP assay was used. In this assay, HEK293 cells were transfected with a cAMP reporter vector pGloSensor^{TM}-22F expressing a cAMP-luciferase reporter protein, which emits light upon binding of cAMP and of the substrate Luciferin. Interestingly, upon short-term treatment with Cur or THC (10 μM) for 0–6 h, THC had a slightly higher ability to increase cAMP levels in HEK293 cells, and CurG was even higher whereas BDCur was lower (Fig. 5A). A robust increase was observed after 2 h with all compounds tested, and occurred at the concentration of at least 2.5 μM (Fig. 5B). Interestingly, after 24 h, Cur was more effective in increasing cAMP levels when compared to THC (Fig. 5C), which is similar to that of the results obtained for CREB-RE activity reporter assay (Fig. 3C), suggesting that differential uptake and metabolism are at the basis of CD36 promoter activity differences seen with Cur and THC after 24 h. By comparing the effect of Cur with one of the known controls, such as the phosphodiesterase (PDE) inhibitor 3-isobutyl-1-methylxanthine (IBMX) and the adenylate cyclase (AC) activators (adenosine, forskolin), it is concluded that the effects of Cur are slower and weaker than adenosine and forskolin but similar in strength as IBMX (Fig. 5D). An analogous although weaker regulatory effect of Cur on cAMP levels and CREB-RE activity was observed in THP-1 monocytes (Supporting Information, Fig. 4).

4. Discussion

The liver is an organ with central metabolic functions, including lipogenesis, gluconeogenesis, and cholesterol synthesis as well as the primary site of metabolism for natural dietary components [60–62]. Macro and micronutrients such as fatty acids

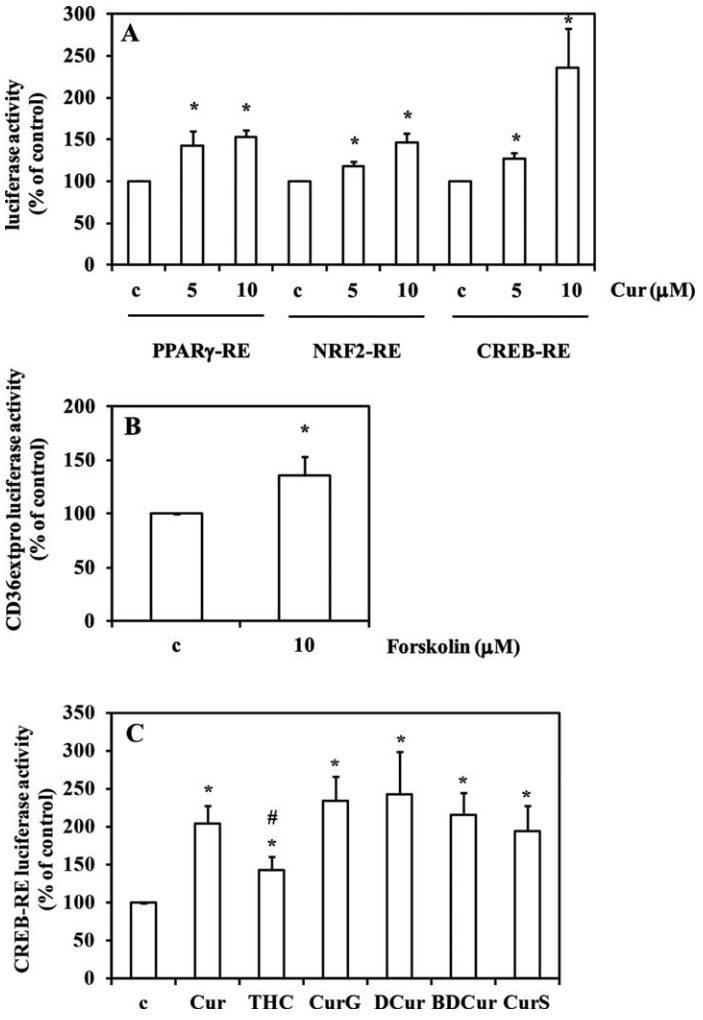

FIG 3

*Cur activates PPARγ, NRF2, and CREB activity in HEK293 cells. (**A**) Treatment with Cur (5 and 10 μM) for 24 h increases PPARγ-RE, NRF2-RE, and CREB-RE, as assessed by transfecting response element luciferase reporter constructs for these transcription factors into HEK293 cells. (**B**) The human CD36 promoter is controlled by cAMP/CREB. HEK293 cells were transfected with a human CD36 promoter luciferase reporter constructs and treated with forskolin (10 μM) for 24 h (n = 4, ±SEM, P < 0.05, with untreated control (c) set to 100%). (**C**) Differential effects of Cur metabolites on CREB-RE luciferase activity. HEK293 cells treated with Cur, THC, CurG, DCur, BDCur, or CurS (all 10 μM) for 24 h and luciferase activity measured (n = 4, ±SEM, *P < 0.05, with untreated control (c) set to 100%, #P < 0.05 relative to Cur).*

and Cur influence the metabolism of the liver and its ability to maintain a normal plasma lipid homeostasis, with consequent lowering the risk of diseases in which hyperlipidemia and lipid-mediated stress play important roles, such as inflammation, steatohepatosis, diabetes, obesity, and atherosclerosis (reviewed in refs. 9,11,63). In fact, the liver has been reported to show the strongest transcriptional response upon supplementation by Cur, whereas in other organs such as intestine, kidney, and spleen, it was weaker or absent [37].

In liver, we found increased CD36 expression in response to an HFD compared to a LFD, and Cur (500 and 1,000 mg/kg diet) slightly increased its expression at the protein level. Although the effects were weak, they might be relevant since they may occur only in a subset of cells in tissues, such as endothelial or epithelial cells that are involved in the uptake of lipids. CD36, also named fatty acids transporter (FAT), is an important regulator of plasma lipid levels [64], as demonstrated in several experimental systems in which the level of expression of CD36 has been modified, for example, by overexpressing CD36 or in CD36 knockout mice. These experiments revealed that the influence of CD36 on plasma lipids depends on the organ in which CD36 was induced or inhibited [65,66], and that some organs/tissues are critically dependent on fatty acids influx by CD36 and for others too much fatty acid influx may not be desirable. It is important to note that patients with CD36 deficiency have increased plasma free fatty acid levels and increased atherosclerotic cardiovascular disease [67], which suggest that CD36-mediated signaling and lipid removal from the circulation is a protective mechanism. In contrast, since heart steady-state energetic metabolism relies mostly on fatty acids, deficiency of CD36 can lead to hypertrophic cardiomyopathy and metabolic syndrome [68]. In the liver, CD36 deletion reduces VLDL secretion and increases hepatic steatosis [69], suggesting that upregulation of CD36 by Cur may have protective effects. The here observed increase of CD36 expression in the liver by HFD and Cur supplementation may particularly be relevant for postprandial uptake of excess fatty acids mediated by insulin-activated Akt/PPARγ/CD36 expression. In the absence of insulin (e.g., diabetes) or during impaired insulin signaling (e.g., insulin resistance), a lower activation of CD36 expression may lead to insufficient removal of plasma lipids with consequent hyperlipidemia, and it remains to be investigated whether Cur can also increase CD36 in this situation [70].

In skeletal muscle, we found that HFD downregulated CD36 expression, which can be considered as protection of muscle against accumulation of lipids when lipids are abundant and not needed, for example, in the absence of increased energy demand induced by exercise, but Cur supplementation still had an inducing effect on CD36 expression. Again, this may help in reducing hyperlipidemia since muscle-specific overexpression of CD36 is associated with lower levels of body fat, serum free fatty acids, and triacylglycerides and higher fasting glucose and insulin levels [71].

Several transcription factors have been shown to be responsive to Cur, and some of them (e.g., PPARγ, SREBP-1/2, NRF2, CREB) have a regulatory role for CD36 expression (reviewed in ref. 11). Using promoter deletion analysis, the regulatory effects of Cur were narrowed down to a 480 bp fragment containing a bona fide CREB response element along with a number of less conserved elements (HIF1α, NRF2, ER, SREBP), but the exact Cur-response element(s) within the

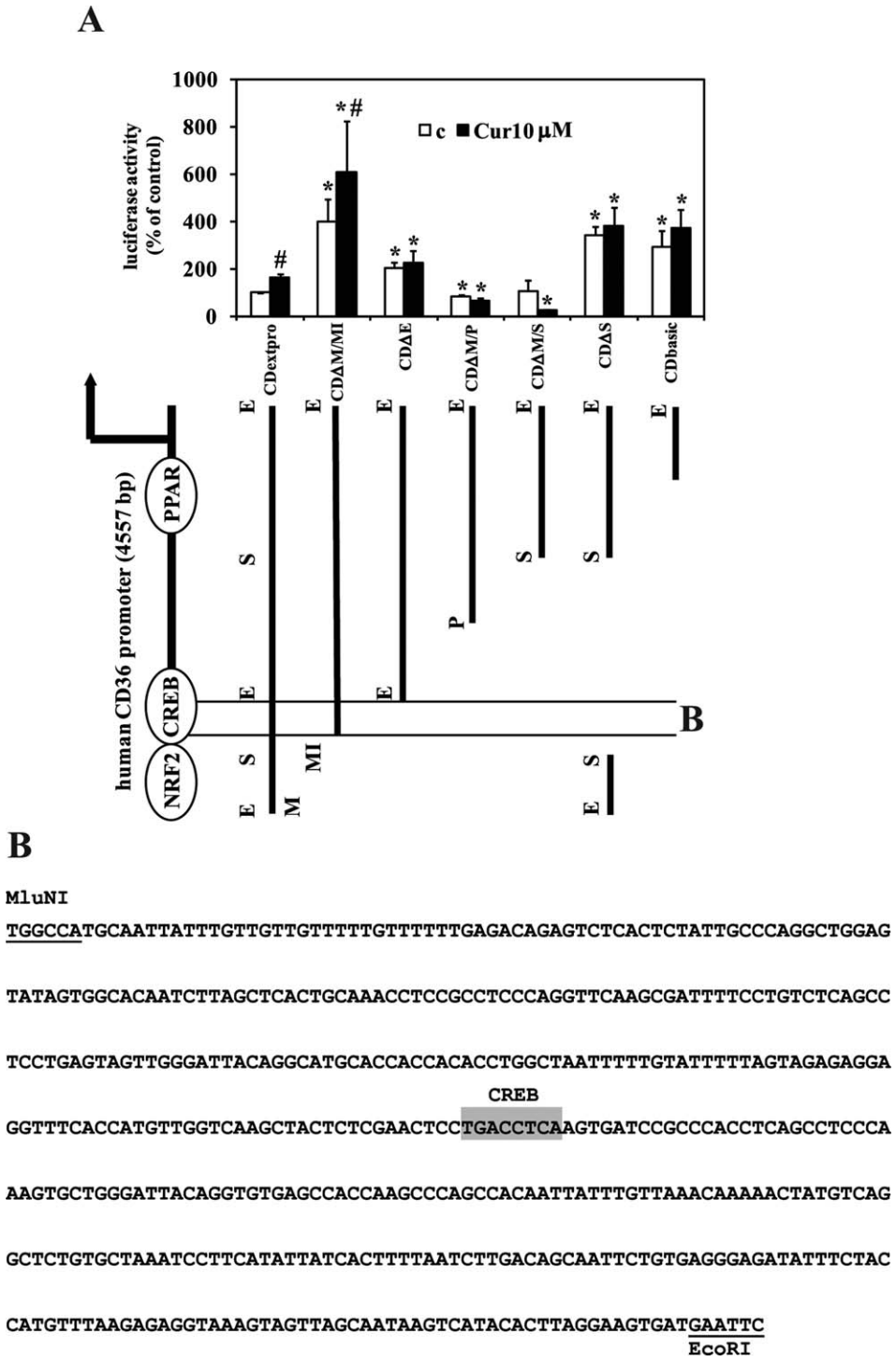

FIG 4

*A distant site of the human CD36 promoter containing a CREB consensus site is responsive to Cur. (**A**) Treatment with Cur (10 μM) for 24 h increases the full length (pCDextpro) and the ΔMlul/MluNI CD36 promoter-luciferase reporter construct (pCDDM/MI), but not any of the other constructs, as assessed by transfection of these constructs into HEK293 cells for 24 h (*n = 6, ±SEM, *$P < 0.05$, with untreated pCDextpro control (c) set to 100%; #$P < 0.05$, compared to transfected plasmid in the absence of Cur). M: MluI, MI: MluNI, E: EcoRI, P: PvuII, S: StuI. (**B**) Sequence of the 480 bp Cur-responsive region and identification of a bona fide consensus regulatory element for CREB.*

human CD36 promoter remain to be determined by a detailed analysis by mutations, footprinting, and chromatin immunoprecipitation. Although Cur could increase PPARγ activity in cell culture, activation of the human CD36 promoter by Cur was independent of PPARγ and FOXO3a activation [8,15], indicating involvement of other transcription factors. In cells, Cur

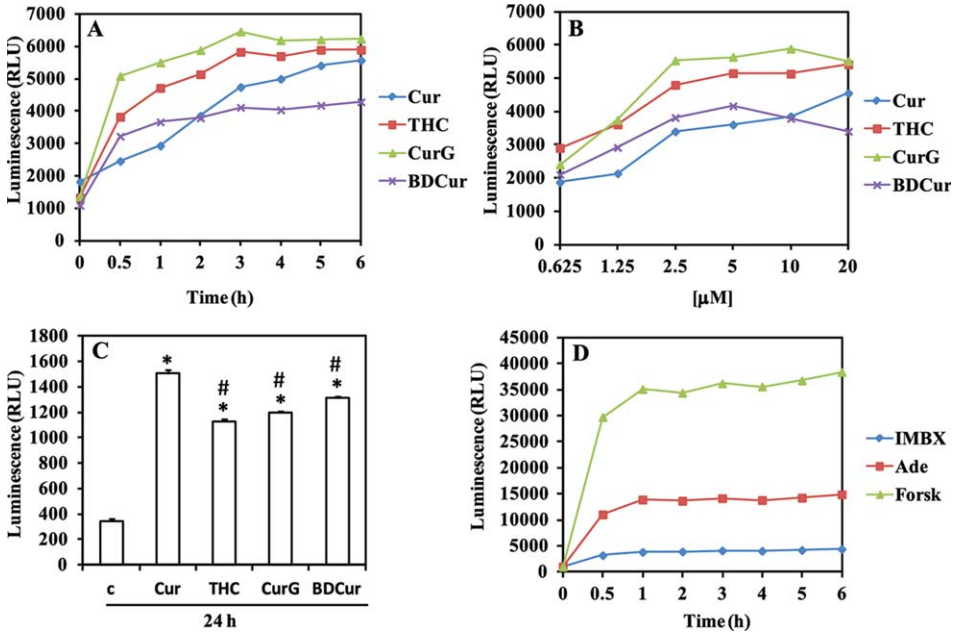

FIG 5

*Regulation of cAMP levels by Cur metabolites. HEK293 cells were transfected with a cAMP reporter vector pGloSensor™-22F expressing a cAMP-luciferase reporter protein, which emits light upon binding cAMP and then treated with Cur, THC, CurG, or BDCur. (**A**) Time dependence of cAMP accumulation after short term treatment (0–6 h) with Cur or Cur metabolites (all 10 μM). (**B**) Concentration dependence of cAMP accumulation after treatment with Cur or Cur metabolites (0.625–20 μM) for 2 h. (**C**) Long-term treatment (24 h) with Cur or Cur metabolites (all 10 μM) (n = 2, ±SEM, *P < 0.05, relative to untreated control; #P < 0.05, relative to treatment with Cur). (**D**) Accumulation of cAMP after treatment for 0–6 h with the adenylate cyclase (AC) activator forskolin (Forsk, 10 μM) or 2′-5′-dideoxyadenosine (Ade, 250 μM), or the phosphodiesterase inhibitor IBMX (25 μM).*

stimulated NRF2, Hif1α, and more strongly CREB activity suggesting that these factors may play a role in upregulating CD36 expression. Moreover, since the response of CD36 to HFD was different in liver and skeletal muscle, tissue-specific regulatory mechanisms may be involved, such as HFD-induced liver CD36 expression as result of PPARγ activation [15,41,42].

At the molecular level, we find in liver and less in adipose tissue (visceral, inguinal subcutaneous) and spleen that HFD significantly decreases and Cur increases tissue cAMP levels, whereas no significant effect was observed in interscapular subcutaneous fat, brain, skeletal muscle, and kidney. We recently reported that Cur is rapidly metabolized in cells [50], which may imply that the regulatory effects on cAMP may be caused not necessarily by Cur only but also by its metabolites. In fact, in HEK293 cells, the increase of cAMP levels occurred within 4 h at which the Cur metabolite THC had the strongest effect, whereas after 24 h (the incubation time used for promoter assays), cAMP levels were highest with Cur rather than Cur metabolites such as THC, CurG, and BDCur. Therefore, delayed uptake and slower metabolism of Cur when compared to THC may be responsible for its higher stimulatory effect on CD36 expression observed at 24 h. The level of cAMP influences the activities of protein kinase A (PKA) and of the transcription factor CREB. These proteins play important roles in lipid homeostasis, energy expenditure, and thermogenesis by increasing lipolysis and fatty acid oxidation through stimulation of hormone sensitive lipase (HSL) and perilipin A [16,17,72]. Thus, according to our data and as outlined in the proposed molecular model (Fig. 6), an increase in cAMP levels induced by Cur could be at the basis of its hypolipidemic and antiatherosclerotic effects and similar to other nutritional supplements such as forskolin or caffeine may also explain its effect on weight loss [20,21,29].

Although it was reported that CREB expression was decreased in wild-type mice after 2 weeks on a HFD [41], in our $Ldlr^{-/-}$ mice treated for 16 weeks, we find slightly elevated CREB phosphorylation in spite of decreased cAMP. CREB phosphorylation was stimulated by Cur probably as a result of increasing cAMP [51,52]). In line with this notion, Cur activated cAMP and CREB activity in cultured cells. CREB is mainly activated by phosphorylation by cAMP-activated protein kinase A (PKA) and subsequent nuclear translocation [73], but alternative activation mechanisms may also be involved such as the EPAC/PI3K/Akt pathway [74]. It remains to be resolved whether Cur increases cAMP similar as resveratrol [28] by inhibiting phosphodiesterases (PDE), or by stimulating adenylate cyclases (AC) activity [54,55]. CREB is also activated by nonesterified fatty acids (NEFA) in vascular smooth muscle cells (VSMC) most likely as a protective response to NEFA-mediated stress [75]. Since CREB is downregulated in aortic VSMC in a high-fat-diet-induced model of vascular disease [73], Cur may normalize CREB and thus be atheroprotective. Accordingly, increased cAMP decreases lipid content in adipocytes and in atherosclerotic lesions [76].

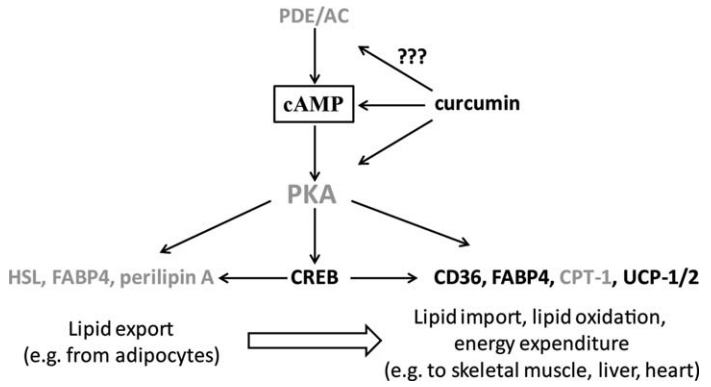

FIG 6

Scheme of proposed molecular mechanisms involved in hypolipidemic effects of Cur. Cur increases cAMP in liver, either by increasing adenylate cyclase (AC) activity or inhibiting phosphodiesterase (PE) activity. Increased levels of cAMP activate PKA/CREB which induces lipid transport proteins (CD36, aP2), and genes involved in lipid metabolism (e.g., HSL, CPT-1) and energy homeostasis (e.g., UCP1/2) leading overall to a hypolipidemic effect. Whether Cur also regulates lipid export from adipose tissue by regulating HSL, aP2, or perilipin 2 remains to be shown (gray letters).

At this time, we do not rule out that some of the effects of Cur on lipid homeostasis may also be mediated by the CREB-related proteins such as cAMP response element-binding protein hepatocyte-specific (CREBH) or cAMP response modulator (CREM), since they also play crucial roles in hepatic lipogenesis, fatty acid oxidation, and lipid metabolism by controlling a number of genes involved in triglyerides metabolism and influence plasma levels of free fatty acids, triglycerides, and cholesterol [46,77,78]. CREBH is activated by metabolic stress induced by saturated fatty acids, insulin signals, or an atherogenic high-fat diet in the liver [79], and Cur reduced endoplasmic reticulum (ER) stress by inhibiting CREBH/CREB3L3 by stimulating the LKB1/AMPK/SMILE/PGC1α pathway [51]. Moreover, an induction of cAMP and CREBH by Cur may not only contribute to its hypolipidemic but also its hypoglycemic effects, e.g., by improving glucose metabolism and insulin resistance [27,80,81].

A Cur-mediated increase of lipids uptake in liver in the absence of lipid accumulation may require increased lipid excretion in bile (e.g., of cholesterol metabolites) (reviewed in ref. 11) or increased channeling into anabolic pathways (e.g., protein and phospholipid synthesis), which is reflected in part by the observed increase in liver size and preservation of glycogen content [10]. Alternatively, Cur may stimulate energy dissipation, for example, by direct uncoupling of mitochondrial oxidative phosphorylation [82], or by upregulation of proteins involved in catabolic metabolism and metabolic inefficiency such as UCP1 [83] and AMPK/UCP2 [84–86]. In fact, UCP1 is regulated by FOXO1 in liver [87] and UCP2 expression is induced by obesity [88] and Cur [89].

Taken together, our *in vivo* data suggest that Cur exerts regulatory effects on cAMP cellular concentrations predominantly in the liver. At this time, we can only correlate the elevated cAMP levels with the *in vivo* Cur-induced hypolipidemic effects, although our *in vitro* experiments suggest a direct cause–effect relationship between Cur, cAMP, and the regulation of genes involved in lipid and energy homeostasis such as CD36, UCP1/2, and aP2. Since the liver centrally participates in the regulation of plasma lipid, cholesterol, and glucose levels [60–62], it remains to be evaluated to what degree the observed effects of Cur in other organs are in fact secondary effects of regulatory events occurring in liver.

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JMZ, RR, AA, and MM designed research, analyzed data, and wrote the article. JMZ, STH, KN, and EC conducted research, and LV provided essential reagents. All authors read and approved the final manuscript. None of the authors has declared any conflict of interest.

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