Glucagon-induced extracellular cAMP regulates hepatic lipid metabolism

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Abstract

Hormonal signals help to maintain glucose and lipid homeostasis in the liver during the periods of fasting. Glucagon, a pancreas-derived hormone induced by fasting, promotes gluconeogenesis through induction of intracellular cAMP production. Glucagon also stimulates hepatic fatty acid oxidation but the underlying mechanism is poorly characterized. Here we report that following the acute induction of gluconeogenic genes *Glucose 6 phosphatase (G6Pase)* and *Phosphoenolpyruvate carboxykinase (Pepck)* expression through cAMP-response element-binding protein (CREB), glucagon triggers a second delayed phase of fatty acid oxidation genes *Acyl-coenzyme A oxidase (Aox)* and *Carnitine palmitoyltransferase 1a (Cpt1a)* expression via extracellular cAMP. Increase in extracellular cAMP promotes PPAR α activity through direct phosphorylation by AMP-activated protein kinase (AMPK), while inhibition of cAMP efflux greatly attenuates *Aox* and *Cpt1a* expression. Importantly, cAMP injection improves lipid homeostasis in fasted mice and obese mice, while inhibition of cAMP efflux deteriorates hepatic steatosis in fasted mice. Collectively, our results demonstrate the vital role of glucagon-stimulated extracellular cAMP in the regulation of hepatic lipid metabolism through AMPK-mediated PPAR α activation. Therefore, strategies to improve cAMP efflux could serve as potential new tools to prevent obesity-associated hepatic steatosis.

Key Words

- ▶ glucagon
- ▶ extracellular cAMP
- ▶ AMPK
- ▶ PPAR α
- ▶ fatty acid oxidation

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Introduction

The expanding global epidemic of obesity has become a major threat to human health worldwide (Ford *et al.* 2004). Obesity is associated with an ectopic accumulation of fat in non-adipose tissues such as in the liver (Sun & Karin 2012). Disrupted lipid metabolism including fatty acid oxidation and *de novo* lipogenesis in liver results in the development of hepatic steatosis and contributes to the development of hepatic insulin resistance (Marchesini *et al.* 2003, Hooper *et al.* 2011). Thus, better understanding of the underlying mechanism and regulation for fatty acid oxidation would greatly help

elucidating novel strategies to treat obesity-induced hepatic steatosis.

The liver is a major organ that controls glucose and lipid metabolism in response to hormonal signals. Dedicated transcription regulatory network is involved in the hepatic response to fasting. During the fasted state, one of the major circulating hormones required for liver function is glucagon. Increased glucagon stimulates the gluconeogenesis via induction of intracellular cAMP. Induction of cAMP-dependent protein kinase A (PKA) by cAMP subsequently leads to activation of the CREB

regulated transcription coactivators (CRTC)/CREB (Altarejos & Montminy 2011) and the Class II histone deacetylases (HDACs)/FOXO pathways (Mihaylova *et al.* 2011, Wang *et al.* 2011) and promotes expression of gluconeogenic genes such as *Pepck* and *G6Pase*. Glucagon also regulates fatty acid oxidation in the liver (Longuet *et al.* 2008, von Meyenn *et al.* 2013). Glucagon stimulation is sufficient to reduce plasma triacylglycerol and non-esterified fatty acid (NEFA) levels and glucagon receptor knockout mice show increased triacylglycerol and NEFA levels during the fasted state (Longuet *et al.* 2008). PPAR α , a master transcription factor that regulates expression of fatty acid oxidation genes such as *Aox*, *Cpt1a* and *Medium-chain acyl-CoA dehydrogenase (Mcad)*, serves as the major target of glucagon's action. Glucagon stimulates PPAR α activity and targets fatty acid oxidation gene expression, which is diminished in PPAR α knockout mice (Longuet *et al.* 2008). Although AMPK is implicated to mediate glucagon-induced PPAR α activity, the underlying mechanism for AMPK activation by glucagon stimulation and AMPK regulation of PPAR α activity remains poorly understood. Glucagon-stimulated gluconeogenesis and fatty acid oxidation are intertwined. The expression of *Pepck* (Tachibana *et al.* 2005, Bernal-Mizrachi *et al.* 2007) and *G6Pase* (Im *et al.* 2011) is also regulated by PPAR α and PPAR α knockout mice are consistently hypoglycemic due to impaired hepatic glucose output. Additionally, *Cpt1a* has been reported to be a CREB target gene (Louet *et al.* 2002).

cAMP has been reported to have both intracellular and extracellular functions. cAMP is secreted from multiple tissues including kidney (Coulson & Bowman 1974), liver (Constantin *et al.* 1997) and adipose tissue (Strouch *et al.* 2005) and cell types such as hepatocytes (Pilkis *et al.* 1975), glomerular epithelial cells (Ardailou *et al.* 1993), renal proximal tubule cells (Boumendil-Podevin & Podevin 1977) and adipocytes (Butcher *et al.* 1968). Extracellular cAMP functions as a paracrine or endocrine molecule to regulate the functions of tissues. Glucagon not only stimulates intracellular cAMP accumulation in the liver, but also induces a significant release of liver-borne cAMP into the blood (Exton *et al.* 1971b, Godinho *et al.* 2015). Extracellular cAMP mediates glucagon actions such as endocrine inhibition of sodium and phosphate reabsorption in the renal proximal tubule (Ahloulay *et al.* 1996). Liver-borne extracellular cAMP is also able to mimic glucagon effect on glucose metabolism in the liver (Northrop & Parks 1964). However, understanding the physiological

function of cAMP *in vivo* and its pathological contribution to metabolic dysfunction in type 2 diabetes (T2DM) still requires further investigation.

Here we report a novel role of glucagon-stimulated extracellular cAMP on fatty acid oxidation and lipid metabolism in the liver. Extracellular cAMP activates hepatic AMPK, which in turn phosphorylates PPAR α and increases its transcriptional activity and fatty acid oxidation gene expression. When administrated *in vivo*, cAMP greatly ameliorates obesity-induced hepatic steatosis. Our data thus provide new evidence demonstrating the effect of extracellular cAMP on hepatic lipid homeostasis under physiological and pathological conditions. Further, we demonstrate a potential novel therapeutic strategy for the treatment of obesity-associated hepatic steatosis.

Materials/subjects and methods

Cells, antibodies and reagents

Primary hepatocytes were prepared as described (Dentin *et al.* 2004). Briefly, livers from fed mice were perfused with Hank's balanced salt solution (Invitrogen) followed by collagenase (Type IV) (Sigma) at a rate of 6 mL/min through the portal vein. Cells were seeded in medium M199 (Invitrogen), supplemented with 0.2% (w/v) bovine serum albumin, and 2% (v/v) fetal bovine serum (FBS). The medium was replaced with fresh M199 medium after 2 h. Cells were then infected with 1 plaque-forming unit per cell (pfu/cell) of Ad-PPAR α , Ad-PPAR α S452A, Ad-AMPK CA or Ad-GFP for 24 h for overexpression and Ad-PPAR α i, Ad-MRP4i or Ad-USi for 48 h for RNAi-mediated knockdown. HEK293T cells were maintained in DMEM with 10% FBS and transfected with Lipofectamine 2000 (Invitrogen). HepG2 cells (human hepatoma cells) were maintained in Ham's F12 with 10% FBS and transfected with FuGENE6 (Roche). Cell treatment information for each experiment was indicated in the figure legend. Anti-pAMPK, AMPK and phosphor-(Ser/Thr) antibodies were purchased from Cell Signaling Technology. Anti- α -tubulin, PPAR α and MRP4 antibodies were purchased from Abcam. cAMP was purchased from Promega. AICAR was purchased from Tocris (Ellisville, MO, USA). Glucagon, Compound C, Probenecid, IMBX, AMPCP, NBTI, DPSPX and GW6471 were purchased from Sigma. Oltipraz was purchased from MedChem Express (Princeton, NJ, USA). Plasmids expressing WT PPAR α , PPAR α S452A and AMPK CA (AMPK T172D)

were provided by Dr Marc Montminy (Salk Institute, La Jolla, USA). RNAi was constructed using the sequence 5'-AGTTGGAGCAGGCAAG-3' for MRP4i and sequence 5'-GGGATCAAAGAGGAGCCAGTGC-3' for PPAR α i.

Animals and adenovirus

C57BL/6J mice and *ob/ob* mice were purchased from Shanghai Laboratory Animal Center (Shanghai, China) and were adapted to colony cages with 12 h light/darkness cycle in a temperature-controlled environment with free access to water and standard irradiated rodent diet (5% fat; Research Diet, D12450, New Brunswick, NJ, USA). For high-fat diet (HFD) studies, 6-week-old mice were maintained on HFD (60% fat; Research Diets, D12492) for 12 weeks. For adenovirus injection, 10^9 pfu Ad-G6Pase-luc or Ad-Aox-luc and 5×10^7 pfu Ad-RSV- β -galactosidase (β -gal) for *in vivo* imaging, 1×10^8 pfu Ad-unspecific RNAi (USi), Ad-MRP4 RNAi (MRP4i) for *in vivo* RNAi-mediated knockdown were delivered by tail vein injection. For cAMP injection, mice were intravenously injected with 20 mg/kg cAMP dissolved in 0.9% saline or 0.9% saline daily for 6 days. After day 6 injection, mice were fasted for 12 h, injected with cAMP and remained fasted for another 12 h before being killed. For AMPCP injection, mice were intravenously injected with 20 mg/kg AMPCP daily for 3 days as previously described (Ponce *et al.* 2016). For olipraz injection, mice were intraperitoneally injected with 150 mg/kg olipraz or coin oil. All animal studies were approved by the Animal Experiment Committee of Tongji University and in accordance with the guidelines of school of medicine, Tongji University. All adenovirus were constructed using AdEasy Adenoviral Vector System according to the manufacturer's instruction (Agilent Technologies).

In vivo imaging

Mice were imaged on Day 3–5 after adenovirus delivery. Mice were fasted for 12 h or 48 h, respectively, and injected intraperitoneally with 50 mg/kg nembutal (Abbott Laboratories) and 100 mg/kg sterile firefly D-luciferin (Qianchen, Shanghai, China) before imaging. Mice were imaged using the IVIS 100 Imaging System (PerkinElmer) and data were analyzed using Living Image software (Xenogen) as described (Liu *et al.* 2008). Liver samples were taken immediately after imaging and lysates were subjected to β -gal assay to ensure the equal level of adenoviral infection.

In vitro analysis

Mouse tissues were frozen in liquid nitrogen and kept at -80°C until further use. Livers were homogenized using tissue homogenizer and further sonicated at 4°C in lysis buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 30 mM sodium pyrophosphate, 30 mM sodium fluoride, 1% Triton-X 100, and protease inhibitor cocktail). Lysates were centrifuged for 10 min at $20,000g$ at 4°C and supernatants were reserved for β -gal activity and protein determination. Serum triacylglycerol, NEFA and total ketones were determined using commercially available kits (WAKO). Liver samples were weighted and incubated overnight at 55°C in ethanolic KOH with vortex. The supernatants were supplied with 50% EtOH and MgCl_2 (Norris *et al.* 2003). After incubation on ice, samples were centrifuged and supernatants were subjected to glycerol determination with WAKO LabAssay Triglycerides kit. Lipids were extracted from liver samples using Lipid Extraction Kit (Sigma) and used for NEFA detection with WAKO LabAssay NEFA kit. Intracellular and extracellular cAMP concentrations were measured using a cAMP ELISA kit according to the manufacturer's instruction (Cayman Chemical Company). Briefly, cell culture media or serum were diluted in ELISA buffer and assayed directly to measure extracellular cAMP levels. To detect intracellular cAMP levels, cultured cells were incubated with 0.1 M HCl at room temperature for 20 min and homogenized by pipetting up and down. After centrifuge, the supernatants were diluted in ELISA buffer and assayed to measure intracellular cAMP levels. The samples of tissues were frozen immediately after collection. Tissues were homogenized in ice-cold 5% trichloroacetic acid (TCA) and centrifuged at $1500g$ for 10 min. TCA in supernatant was extracted using water-saturated ether and the aqueous layer was heated to 70°C to remove trace amount of ether. The supernatants were assayed directly to measure intracellular cAMP levels. Serum insulin and glucagon were measured using ELISA kits from Crystal Chem (Downers Grove, IL, USA).

Quantitative real-time PCR and immunoblot

Real-time PCR was performed as previously (Lv *et al.* 2017). Briefly, total RNA was isolated using TRIzol reagent (Invitrogen) and reverse transcription was done using FastQuant RT kit (Tiangen, Shanghai, China). Real-time PCR was carried out using SuperReal SYBR Green kit (Tiangen) and Lightcycler 96 (Roche). All reactions were performed in duplicate. The amplification efficiency

for each primer pair and the cycle threshold (Ct) were determined automatically by Lightcycler software (Roche). The fold-change was calculated by the comparative CT ($2^{-\Delta\Delta CT}$) method against β -actin (Schmittgen & Livak 2008). Immunoblot and immunoprecipitation were performed as described (Luan *et al.* 2014). Briefly, cells were washed with PBS and then resuspended in lysis buffer (150mM NaCl, 1% Triton-X 100, 1 mM EDTA, 50mM Tris pH7.5, and protease inhibitor cocktail). For immunoblot, protein content in the supernatant was determined using the Micro BCA protein assay kit (Pierce) and suspended in sample buffer (100mM Tris, pH 6.8, 4% SDS, 20% glycerol, 0.1% bromophenol blue). The samples were separated on SDS-PAGE gels, transferred, probed with antibodies and visualized using ECL reagents. For immunoprecipitation, the supernatant was precleaned with protein A/G agarose for 30min and then incubated overnight on a rocker with primary antibodies at 4°C, followed by incubation with Protein A/G agarose beads for another 2h. The immunoprecipitates were extensively washed with lysis buffer and suspended in sample buffer for SDS-PAGE analysis. The immunoreactive bands were quantified with Image-Pro Plus software (Media Cybernetic, Chicago, IL, USA), and quantification using Sigma Plot 8.0 (Systat Software, Chicago, IL, USA) was based on at least three independent experiments.

Primer sequences used for real-time PCR:

Pepck forward 5'-CTGCATAACGGTCTGGACTTC-3'
Pepck reverse 5'-CAGCAACTGCCCGTACTCC-3'
G6Pase forward 5'-ACTGTGGGCATCAATCTCCTC-3'
G6Pase reverse 5'-CGGGACAGACAGACGTTACGC-3'
Pgc1a forward 5'-CCCTGCCATTGTTAAGACC-3'
Pgc1a reverse 5'-TGCTGCTGTTCTGTTTC-3'
 β -*Actin* forward 5'-GTGACGTTGACATCCGTAAAGA-3'
 β -*Actin* reverse 5'-GCCGGACTCATCGTACTCC-3'
Aox forward 5'-AGTTCCATGACCCATCTCTGTC-3'
Aox reverse 5'-CAGCAACTGCCCGTACTCC-3'
Cpt1a forward 5'-CTCCGCCTGAGCCATGAAG-3'
Cpt1a reverse 5'-CACCAGTGATGATGCCATTCT-3'
Mcad forward 5'-AGGGTTTAGTTTGTAGTTGACGG-3'
Mcad reverse 5'-CCCCGCTTTTGTATATTCG-3'
Ppara forward 5'-AGAGCCCCATCTGTCTCTC-3'
Ppara reverse 5'-ACTGGTAGTCTGCAAAACCAA-3'
Cyp4a10 forward 5'-TTCCTGATGGACGCTCTTA-3'
Cyp4a10 reverse 5'-GCAACCTGGAAGGGTCAAAC-3'
Cyp4a14 forward 5'-TTAGCCCTACAAGGTACTTGGA-3'
Cyp4a14 reverse 5'-GCAGCCACTGCCTTCGTAA-3'
Hmgcl forward 5'-CAGGTGAAGATCGTGGAAGTC-3'
Hmgcl reverse 5'-GGAGCCCTGCTTCGGAAAC-3'

Hmgcs2 forward 5'-GAAGAGAGCGATGCAGGAAAC-3'
Hmgcs2 reverse 5'-GTCCACATATTGGGCTGGAAA-3'

Luciferase reporter assay

Primary hepatocytes were infected with 1pfu/cell of Ad-*G6Pase*-luc or Ad-*Aox*-luc adenovirus together with Ad-RSV- β -gal adenovirus for 24h and luciferase assays were performed using Promega GloMax96 system according to the manufacturer's instruction (Luan *et al.* 2014). β -Gal assay was used to normalize the expression levels.

Liver histological analysis

Sections (5 μ m) of formalin-fixed paraffin-embedded mouse liver were used for haematoxylin and eosin (H&E) staining. Livers embedded in optimal cutting temperature compound (Tissue-Tek, Laborimpex) were used for Oil Red O staining to assess hepatic steatosis.

Glucose tolerance test (GTT) and insulin tolerance test (ITT)

For glucose tolerance testing, mice were fasted for 16h, and then injected intraperitoneally with glucose (1.5g/kg). For insulin tolerance testing, mice were fasted for 2h, and then injected intraperitoneally with insulin (Humulin; 1 U/kg). Blood was collected from the tail vein at indicated times and glucose levels were measured using a One Touch Ultra Glucometer (Johnson & Johnson).

Statistical analysis

All studies were performed on at least three independent occasions. Results are reported as mean $\pm$ S.E.M. Differences between two groups were assessed using unpaired Student's *t* test. Data involving more than two groups were assessed by analysis of variance (ANOVA) with Bonferroni *post hoc* test. A *P*-value of <0.05 was considered statistically significant.

Results

Glucagon differently stimulates gluconeogenesis and fatty acid oxidation

To monitor glucagon's effect on hepatic gluconeogenesis and fatty acid oxidation *in vivo*, we delivered *G6Pase*-luc or *Aox*-luc adenovirus in mouse liver via tail vein

injection and imaged their luciferase reporter activities, respectively, under fasting conditions. *G6Pase-luc* activity but not *Aox-luc* activity increased remarkably in the liver after 12h fasting (Fig. 1A). On the contrary, *Aox-luc* activity but not *G6Pase-luc* activity increased after 48h fasting (Fig. 1A). While 12h fasting increased serum glucagon and cAMP levels as expected, 48h fasting further boosted their secretion (Fig. 1B). Meanwhile, serum triacylglycerol levels were further decreased in 48h fasted mice in comparison with 12h fasted mice; while ketone bodies increased more dramatically in 48h fasted mice in comparison with 12h fasted mice (Fig. 1B). Similarly, the expression of endogenous gluconeogenic genes including *Pepck*, *G6Pase* and *Peroxisome proliferator-activated receptor γ coactivator 1- α* (*Pgc1a*) and fatty acid oxidation genes including *Cpt1a*, *Aox* and *Mcad* was also differently induced by fasting in the liver (Fig. 1C).

Furthermore, exposure to glucagon increased the expression of CREB target genes including *Pepck*, *G6Pase*, *Insulin receptor substrate 2* (*Irs2*), *Nuclear receptor subfamily 4 group a member 2* (*Nr4a2*) and *Activating transcription factor 3* (*Atf3*) in primary hepatocytes within 4h but less so after 16h (Supplementary Fig. 1A and B, see section on supplementary data given at the end of this article). By contrast, the expression of PPAR α target genes including *Cpt1a*, *Aox*, *3-Hydroxy-3-methylglutaryl-CoA synthase 2* (*Hmgcs2*), *Mcad* and *Fibroblast growth factor 21* (*Fgf21*) was low at early time (4h) but increased dramatically during late time (16h and 24h) (Supplementary Fig. 1C and D). Consistently, short-time treatment of glucagon-induced *G6Pase-luc* activity but not *Aox-luc* activity, while long-time treatment of glucagon greatly induced *Aox-luc* activity in primary hepatocytes (Supplementary Fig. 1E).

Figure 1

Glucagon differentially stimulates gluconeogenesis and fatty acid oxidation pathway. (A) Effect of short-term and long-term fasting on hepatic *G6Pase* luciferase reporter activity as well as *Aox* luciferase reporter activity in mouse liver. Significance was determined by Student's *t* test (* P < 0.05 compared with 12h fasted mice, n = 8). (B) Effect of short-term and long-term fasting on serum amounts of glucagon, cAMP, triacylglycerol and ketones. Significance was determined by one-way ANOVA (* P < 0.05 compared with *ad libitum* mice; # P < 0.05 compared with 12h fasted mice, n = 8). (C) Effect of short-term and long-term fasting on mRNA amounts for gluconeogenic genes including *Pepck*, *G6Pase* and *Pgc1a* and fatty acid oxidation genes including *Cpt1a*, *Aox* and *Mcad* in mouse liver. Significance was determined by one-way ANOVA (* P < 0.05, ** P < 0.01 compared with *ad libitum* mice; # P < 0.05 compared with 12h fasted mice, n = 8).

Intracellular and extracellular cAMP stimulates gluconeogenesis and fatty acid oxidation, respectively

Binding of glucagon to its receptor leads to the activation of stimulatory G protein (Gs) and elevation of intracellular cAMP levels resulting from activation of adenylyl cyclase. In the meantime, cAMP could also be secreted out of the cells and function as a paracrine or endocrine molecule (Northrop & Parks 1964, Ahloulay *et al.* 1996). Exposure of primary hepatocytes to glucagon triggered a rapid increase in intracellular cAMP levels at early times and increased extracellular cAMP levels at late times, which correlates well with glucagon's effect on gluconeogenic and fatty acid oxidation gene expression (Fig. 2A and Supplementary Fig. 2A). We wondered whether the slow accumulation of extracellular cAMP accounted for the delayed kinetics of fatty acid oxidation gene expression, while intracellular cAMP mainly functioned through induction of gluconeogenesis at early times. We increased intracellular and extracellular cAMP levels by exposure of primary hepatocytes to 3-Isobutyl-1-Methylxanthine (IBMX, a broad-spectrum phosphodiesterase inhibitor) (Fig. 2B and Supplementary Fig. 2B). IBMX greatly increased intracellular and extracellular cAMP levels after glucagon treatment (Fig. 2B and Supplementary Fig. 2B). The expression of *Aox* and *Cpt1a* together with *G6Pase* and *Pepck* was enhanced by IBMX treatment (Fig. 2C and Supplementary Fig. 2C). Similar results were obtained when primary hepatocytes were infected with adenovirus encoding adenylyl cyclase 6 (Ad-AC6) (Fig. 2D, E and Supplementary Fig. 2D, E).

MRP4 has been reported to mediate the efflux of cAMP (Godinho *et al.* 2015). Indeed, when long-term glucagon-stimulated cAMP export in primary hepatocytes was blocked by probenecid (a reported MRP4 inhibitor), extracellular cAMP levels were dramatically lower (Fig. 2F), while intracellular cAMP levels were enhanced (Supplementary Fig. 2F). Consistently, chronic glucagon-stimulated *Aox* and *Cpt1a* expression was blocked by probenecid (Fig. 2G), while *G6Pase* and *Pepck* expression was increased (Supplementary Fig. 2G). Furthermore, when hepatic MRP4 was knocked down by adenovirus mediated MRP4 RNAi (Ad-MRP4i), extracellular cAMP as well as chronic glucagon-stimulated *Aox* and *Cpt1a* expression was dramatically downregulated (Fig. 2H and I), while intracellular cAMP as well as *G6Pase* and *Pepck* expression were upregulated (Supplementary Fig. 2H, I and J). Further, inhibition of glucagon-stimulated *Aox* and *Cpt1a* expression by probenecid was lost in Ad-MRP4i infected cells (Supplementary Fig. 2K). These results indicate that

extracellular cAMP mediates glucagon-stimulated fatty acid oxidation genes expression. To directly test the role of extracellular cAMP, primary hepatocytes were treated with increasing concentrations (10, 50, or 100 μ mol/L) of cAMP with or without glucagon. Consequent expression of *Aox*, *Cpt1a* and *Mcad* increased in a dose-dependent manner (Fig. 2J). Collectively, these data show that extracellular cAMP secreted from hepatocytes mediates glucagon's late phase effect on fatty acid oxidation genes expression.

Extracellular cAMP activates AMPK to promote PPAR α activation

The extracellular cAMP-adenosine pathway has been shown to be present in multiple tissues (Rosenberg *et al.* 1994, Dubey *et al.* 1996, 2000, Jackson & Mi 2000). Extracellular cAMP is metabolized to AMP and adenosine in plasma membranes by ecto-phosphodiesterase and 5'-nucleotidase (5'-NT), respectively (Jackson 1991). Based on the ability for adenosine to activate AMPK kinase through adenosine transporter CNT2 (Aymerich *et al.* 2006), we examined whether extracellular cAMP was able to activate this pathway in primary hepatocytes. After 24 h cAMP exposure in primary hepatocytes, phosphorylation of AMPK increased in a concentration dependent manner (Fig. 3A). Consistent with the role of glucagon on cAMP efflux, chronic treatment with glucagon also increased phosphorylation of AMPK (Fig. 3B); this effect was blocked by either probenecid or Ad-MRP4i infection (Fig. 3C). Furthermore, α , β -methylene adenosine 5-diphosphate (AMPCP, a 5'-NT inhibitor), S-(4-nitrobenzyl)-6-thioinosine (NBTI, an adenosine concentrative transporter CNT2 inhibitor), but not 1, 3-dipropyl-8-p-sulfophenylxanthine (DPSPX, a pan adenosine receptor antagonist) ameliorated glucagon's effect on AMPK phosphorylation (Fig. 3D). Exposure of primary hepatocytes to 5-Aminoimidazole-4-carboxamide-1- β -D-ribofuranoside (AICAR, an AMPK activator) mimicked the effect of extracellular cAMP on *Aox* and *Cpt1a* expression (Fig. 3E). These results suggest that extracellular cAMP-adenosine pathway activates AMPK through adenosine transporter but not adenosine receptor in hepatocytes.

To test the requirement of AMPK for the effect of extracellular cAMP on fatty acid oxidation genes expression, primary hepatocytes were incubated with compound C (an AMPK inhibitor). Compound C inhibited glucagon or cAMP-induced *Aox* and *Cpt1a* expression (Fig. 3F). Consistent with the influence on AMPK phosphorylation, AMPCP and NBTI but not DPSPX

Figure 2

Extracellular cAMP stimulates hepatic fatty acid oxidation. (A) Effect of glucagon (20 nmol/L) stimulation on extracellular cAMP levels at different time point in primary hepatocytes. Significance was determined by one-way ANOVA (* P < 0.05, ** P < 0.01 compared with respective controls, n = 6). (B) Effect of IBMX (0.1 mmol/L) exposure on glucagon-induced extracellular cAMP levels at different time point in primary hepatocytes. Primary hepatocytes were pretreated with IBMX for 1 h and followed by glucagon stimulation for indicated times. Significance was determined by Student's t test (* P < 0.05 compared between indicated groups, n = 6). (C) Effect of IBMX (0.1 mmol/L) exposure on glucagon-induced *Cpt1a* and *Aox* mRNA amounts at indicated time in primary hepatocytes. Significance was determined by Student's t test (* P < 0.05 compared between indicated groups, n = 6). (D) Effect of Ad-AC6 on glucagon-induced extracellular cAMP levels. Primary hepatocytes were infected with Ad-GFP or Ad-AC6 for 24 h and followed by glucagon stimulation for indicated times. Significance was determined by Student's t test (* P < 0.05 compared between indicated groups, n = 6). (E) Effect of Ad-AC6 on glucagon-induced *Cpt1a* and *Aox* mRNA amounts at indicated time in primary hepatocytes. Significance was determined by Student's t test (* P < 0.05 compared between indicated groups, n = 6). (F) Effect of probenecid (500 μ mol/L) exposure on glucagon-induced extracellular cAMP levels at different time point in primary hepatocytes. Primary hepatocytes were pretreated with probenecid for 1 h and followed by glucagon stimulation for indicated times. Significance was determined by Student's t test (* P < 0.05 compared between indicated groups, n = 6). (G) Effect of probenecid (500 μ mol/L) exposure on glucagon-induced *Cpt1a* and *Aox* mRNA amounts at indicated time in primary hepatocytes. Significance was determined by Student's t test (* P < 0.05 compared between indicated groups, n = 6). (H) Effect of Ad-MRP4i on glucagon-induced extracellular cAMP levels. Primary hepatocytes were infected with Ad-USi or Ad-MRP4i for 48 h and followed by glucagon stimulation for indicated times. Significance was determined by Student's t test (* P < 0.05 compared between indicated groups, n = 6). (I) Effect of Ad-MRP4i on glucagon-induced *Cpt1a* and *Aox* mRNA amounts at indicated time in primary hepatocytes. Significance was determined by Student's t test (* P < 0.05 compared between indicated groups, n = 6). (J) Effect of cAMP (10, 50, or 100 μ mol/L) exposure on glucagon-induced *Cpt1a*, *Aox* and *Mcad* mRNA amounts in primary hepatocytes. Primary hepatocytes were pretreated with indicated amount of cAMP for 1 h and followed by control or glucagon stimulation for 24 h. Significance was determined by one-way ANOVA (* P < 0.05 compared with the control only; # P < 0.05 compared with glucagon only, n = 6).

abolished the effect of cAMP and glucagon on *Aox* and *Cpt1a* expression (Fig. 3G and Supplementary Fig. 3).

AMPK is a highly conserved energy sensor that specifically phosphorylates substrates with a consensus motif of (L/M/I)-X-R/K-X-X-(S/T)-X-X-X-L (Gwinn *et al.* 2008). PPAR α master regulator of fatty acid oxidation

contains a conserved AMPK phosphorylation motif (Ser452) (Fig. 4A). We hypothesized that AMPK might increase fatty acid oxidation genes expression through direct phosphorylation of PPAR α . Exposure of primary hepatocytes to AICAR greatly increased ser/thr phosphorylation of endogenous PPAR α (Fig. 4B).

To determine that the phosphorylation of PPAR α was dependent on the AMPK consensus site, HEK293T cells were transfected with WT PPAR α or PPAR α S452A with constitutively active form of AMPK (AMPK CA, AMPK T172D). AMPK consensus site (S452) mutation completely disrupted PPAR α phosphorylation by AMPK CA (Fig. 4C). Further, AICAR or cAMP was able to induce AMPK-mediated phosphorylation of PPAR α but not PPAR α mutant (S452A, Fig. 4D). Expression of constitutively active AMPK (AMPK CA) promoted WT PPAR α activity but not PPAR α S452A activity in inducing *Aox*-luc reporter activity (Fig. 4E) in primary hepatocytes. Consistently, when primary hepatocytes were expressed with PPAR α or PPAR α S452A using adenovirus, the effect of cAMP or AICAR on glucagon-induced *Aox* and *Cpt1a* expression was further improved by PPAR α but not PPAR α mutant (S452A) (Fig. 4F).

To demonstrate whether PPAR α is required for the effects of extracellular cAMP on fatty acid oxidation genes expression, primary hepatocytes were either infected with PPAR α RNAi adenovirus (Ad-PPAR α i) or incubated with 10 μ M of PPAR α antagonist GW6471 and the effect of cAMP on glucagon-induced *Aox* and *Cpt1a* expression was largely abolished (Supplementary Fig. 4A, B and C), suggesting a critical role of PPAR α in cAMP-mediated fatty acid oxidation genes expression.

Extracellular cAMP maintains hepatic lipid homeostasis under fasted stage

The regulation of PPAR α by extracellular cAMP in primary hepatocytes suggests that modulation of extracellular cAMP may influence hepatic lipid homeostasis *in vivo*. It is reported that prolonged fasting induces lipids deposition in the liver (Li *et al.* 2014), prompting us to evaluate the function of extracellular cAMP on lipid homeostasis under fasted state. Indeed, 6 days of intravenous injection of cAMP decreased triacylglycerol and NEFA levels in serum and liver, and increased serum ketone bodies after 24h fasting (Fig. 5A). Although blood glucose levels remained unchanged after cAMP injection, insulin levels decreased dramatically in cAMP injected mice, suggesting improved insulin sensitivity (Fig. 5A). Mice injected with cAMP showed less lipid accumulation in liver compared with controls (Fig. 5B) and expression of PPAR α target genes known to regulate fatty acid oxidation and ketogenesis were significantly increased in livers of mice injected with cAMP (Fig. 5C). By contrast with the effect of cAMP injection, decreasing extracellular cAMP by Ad-MRP4i

infection in liver greatly increased serum and liver triacylglycerol and NEFA levels under 24h fasted state (Fig. 5D). Insulin levels were increased while blood glucose remained unchanged (Fig. 5D). Lipid accumulation was also enhanced (Fig. 5E). The expression of hepatic fatty acid oxidation and ketogenesis genes was decreased in Ad-MRP4i injected mice compared with Ad-USi injected mice (Fig. 5F). Consistent with the influence on AMPK phosphorylation and cAMP-induced *Aox* and *Cpt1a* expression, intravenous AMPCP injection increased triacylglycerol levels in mouse liver (Supplementary Fig. 5A), while it decreased the expression of hepatic fatty acid oxidation and ketogenesis genes after 24h fasting (Supplementary Fig. 5B).

Extracellular cAMP attenuate hepatic steatosis in obese mice

To further examine the role of extracellular cAMP during *in vivo* pathogenesis, mice were grouped and fed either a regular diet (RD) or a HFD for 12 weeks. Hepatic cAMP levels were significantly increased in HFD-fed mice compared with RD-fed mice, while serum cAMP levels were contrarily decreased (Fig. 6A), indicating impaired hepatic cAMP efflux. Indeed, expression of hepatic *Mrp4* was decreased in HFD-fed mice compared with RD-fed mice (Fig. 6B). Similar results were also observed in *ob/ob* mice comparing with lean mice (Supplementary Fig. 6). Changes in extracellular cAMP levels in HFD-fed and *ob/ob* mice indicate the pathophysiological role of extracellular cAMP under diabetic conditions and prompt further investigation into its contribution to obesity-associated hepatic steatosis. Indeed, 6 days of cAMP injection dramatically decreased liver and serum triacylglycerol and NEFA levels in comparison with controls (Fig. 6C). Serum ketone bodies were dramatically increased in mice injected with cAMP compared with controls (Fig. 6C), correlating with increased ketogenesis and fatty acid oxidation by extracellular cAMP in hepatocytes. cAMP injection showed dramatically less lipid accumulation in liver compared with controls evidenced by H&E and Oil Red O staining (Fig. 6D). Consistently, expression of PPAR α target genes known to regulate fatty acid oxidation and ketogenesis were significantly increased in livers of mice injected with cAMP (Fig. 6E). Further, cAMP injection also decreased blood glucose, insulin levels and HFD-induced glucose intolerance and insulin resistance (Fig. 6F and G), indicating insulin-sensitizing effect of cAMP.

Increasing hepatic *Mrp4* expression ameliorates hepatic steatosis caused by high-fat diet

Based on the observation that hepatic *Mrp4* expression was decreased in HFD-fed mice, we hypothesized that increasing hepatic MRP4 expression could ameliorates hepatic steatosis caused by HFD. It has been reported that *Mrp4* expression could be regulated by farnesoid X receptor (FXR) (Schuetz *et al.* 2001), constitutive androstane receptor (CAR) (Aleksunes & Klaassen 2012) and Nuclear Factor-E2-Related Factor-2 (NRF2) (Maher *et al.* 2007). Indeed, hepatic mRNA levels of *Car* and *Nrf2* but not *Fxr* were dramatically reduced in both HFD-fed mice and *ob/ob* mice, suggesting their contribution to the reduced *Mrp4* expression (Supplementary Fig. 7A and B). Oltipraz (OPZ) has been reported to be an activator of both CAR and NRF2 (Merrell *et al.* 2008). When HFD-fed mice were injected with OPZ, reduced *Mrp4* mRNA levels as well as serum cAMP levels were rescued (Supplementary Fig. 7C and D), indicating that reduced hepatic *Car* and *Nrf2* expression in HFD-fed mice account for the reduced *Mrp4* expression and cAMP efflux from the liver. As a result, hepatic triacylglycerol was also decreased in OPZ injected mice (Supplementary Fig. 7E). Additionally, the expression of PPAR α target genes known to regulate fatty acid oxidation and ketogenesis were significantly increased in livers of mice injected with OPZ (Supplementary Fig. 7F). These results suggested that increased *Mrp4* expression was able to improve the hepatic steatosis induced by HFD.

Discussion

Fatty acid oxidation and subsequent ketogenesis is one of the major mechanisms to maintain hepatic lipid homeostasis under fasting conditions. Hormone glucagon has been shown to stimulate ketone body production through activation of PPAR α . Studies using glucagon receptor knockout mice and PPAR α knockout mice have demonstrated the central importance of the glucagon-PPAR α signaling pathway for the control of hepatic lipid metabolism in response to fasting (Longuet *et al.* 2008, von Meyenn *et al.* 2013). However, the molecular basis of this link is still largely undiscovered. In our study, we show that glucagon stimulates PPAR α activity through extracellular cAMP-mediated AMPK phosphorylation, elucidating the signaling cascade that mediates glucagon-stimulated PPAR α activation (Fig. 6H). Most importantly, the administration of cAMP *in vivo* decreased serum and hepatic triacylglycerol, NEFA levels and increased

ketone bodies under fasted stage or following exposure to a HFD, while blocking cAMP efflux from liver by MRP4 knockdown leads to hepatic steatosis. Hence, both *in vitro* and *in vivo* results implicate an essential physiological role of extracellular cAMP in the control of fasting-enhanced fatty acid oxidation.

It is well documented that glucagon-induced efflux of cAMP is also regulated by insulin (Exton *et al.* 1971a, Zumstein *et al.* 1974). Insulin decreases the efflux of cAMP in response to glucagon in hepatocytes (Pilkis *et al.* 1975, Marchmont & Houslay 1980). Thus, the level of extracellular cAMP depends on the ratio between glucagon and insulin concentrations in normal condition. However, in obese mice, which are characterized by hyperglucagonemia and hyperinsulinemia, serum cAMP levels were conversely decreased, due to the decreased expression of *Mrp4* in liver and probably also due to the hyperinsulinemia-induced degradation. Hence, defective glucagon and insulin action coordinates through extracellular cAMP to cause the dysfunction of hepatic fatty acid oxidation which leads to steatosis in the pathophysiology of type 2 diabetes (Unger & Cherrington 2012). Taken into consideration the hyperglycemia caused by elevated glucagon-stimulated glucose production and defective insulin mediated suppression of glucose production due to insulin resistance (Edgerton & Cherrington 2013), our current data thus provide new insight into the understanding of the glucose and lipid defect in subjects with type 2 diabetes through glucagon and insulin together.

Adenosine and its receptors have been shown to play an important role in the pathogenesis of alcoholic fatty liver. Chronic alcohol-stimulated adenosine release stimulates adenosine A₁ and A_{2B} receptors, which promote the development of fatty liver. Blockade or deletion of these receptors diminishes hepatic triglyceride accumulation and development of fatty liver (Peng *et al.* 2009). The function of adenosine and its receptors in nonalcoholic fatty liver is still elusive. Caffeine, a nonselective adenosine receptor antagonist significantly decreases hepatic lipid content in diabetic KK mice (Muroyama *et al.* 2003, Murosaki *et al.* 2007). Most importantly, caffeine intake shows a potential protective effect in patients with nonalcoholic fatty liver disease (Birerdinc *et al.* 2012). Our data also demonstrate that adenosine transporter CNT2 inhibitor NBTI ameliorated glucagon-stimulated fatty acid oxidation genes express. Thus, adenosine receptors may also play a role in the pathogenesis of nonalcoholic fatty liver which will need further investigation.

Figure 4

AMPK phosphorylation promotes PPAR α activation. (A) Conserved AMPK consensus site on PPAR α . (B) Immunoblot showing effects of 2 h AICAR (500 μ mol/L) treatment on PPAR α phosphorylation in primary hepatocytes. Immunoblot intensities for pPPAR α /PPAR α were quantitated. Significance was determined by Student's *t* test (**P* < 0.05 compared with control, *n* = 4). (C) Immunoblot showing effect of mutations in the AMPK phosphorylation site (S452A) on PPAR α phosphorylation by AMPK CA in HEK293T cells. HEK293T cells were transfected with indicated plasmid for 24 h. Immunoblot intensities for pPPAR α /PPAR α were quantitated. Significance was determined by one-way ANOVA (**P* < 0.05 compared with control in PPAR α WT, *n* = 4). (D) Immunoblot showing effect of 2 h AICAR (500 μ mol/L) or 24 h cAMP (100 μ mol/L) treatment on PPAR α or PPAR α S452A phosphorylation by AMPK in HepG2 cells. HepG2 cells were transfected with indicated plasmid for 48 h before treatment. Immunoblot intensities for pPPAR α /PPAR α were quantitated. Significance was determined by one-way ANOVA (**P* < 0.05, compared between indicated groups, *n* = 4). (E) Effect of wild-type or S452A mutant PPAR α on Aox-luc reporter activity. Primary hepatocytes were infected with Ad-Aox-luc, Ad-RSV- β -gal together with Ad-PPAR α , Ad-PPAR α S452A and Ad-AMPK CA adenovirus for 24 h. Significance was determined by one-way ANOVA (**P* < 0.05, compared between indicated groups, *n* = 6). Effect of AMPK CA expression was shown. (F) Effect of wild-type or S452A mutant PPAR α on cAMP (100 μ mol/L)-induced or AICAR (500 μ mol/L)-induced *Cpt1a* and *Aox* mRNA amounts in primary hepatocytes. Primary hepatocytes were infected with Ad-GFP, Ad-PPAR α or Ad-PPAR α S452A for 24 h. Cells were pretreated with cAMP or AICAR for 1 h and followed by glucagon stimulation for 24 h. Significance was determined by two-way ANOVA (**P* < 0.05, GFP vs PPAR α WT, *n* = 6).

Figure 5

Extracellular cAMP maintains lipid homeostasis under fasted stage. (A) Effect of cAMP (20 mg/kg) injection on hepatic and serum triacylglycerol, NEFA, serum ketones, blood glucose and insulin levels in 24h fasted mice. Significance was determined by Student's *t* test (**P* < 0.05 compared with control, *n* = 8). (B) Representative H&E and Oil Red O staining of liver from cAMP injected mice (scale bar, 50 μ m). (C) Effect of cAMP (20 mg/kg) injection on hepatic fatty acid oxidation gene expression. Significance was determined by Student's *t* test (**P* < 0.05, ***P* < 0.01 compared with control, *n* = 8). (D) Effect of Ad-MRP4i injection on hepatic and serum triacylglycerol, NEFA, serum ketones, blood glucose and insulin levels in 24h fasted mice. Significance was determined by Student's *t* test (**P* < 0.05 compared with USi, *n* = 8). (E) Representative H&E and Oil Red O staining of liver from Ad-MRP4i injected mice (scale bar, 50 μ m). (F) Effect of Ad-MRP4i injection on hepatic fatty acid oxidation gene expression. Significance was determined by Student's *t* test (**P* < 0.05, ***P* < 0.01 compared with USi, *n* = 8).

AMPK is a key regulator of fatty acid oxidation in liver. Although AICAR (AMPK activation) treatment alone is enough to cause PPAR α phosphorylation, AICAR alone without glucagon is not enough to increase fatty acid oxidation genes expression (Fig. 3E), suggesting other aspects of the glucagon signaling pathway are required.

Von Meyenn and coworkers have shown that glucagon stimulation induced Lys259 acetylation of Foxa2, which plays an important role in glucagon-stimulated fatty acid oxidation (von Meyenn *et al.* 2013). Our previous work also pointed out that glucagon could promote fatty acid oxidation through releasing the inhibitory effect of SIK2

Figure 6

cAMP administration attenuates hepatic steatosis caused by HFD. (A) Hepatic and serum cAMP levels in RD or HFD-fed mice. Significance was determined by Student's *t* test (* P < 0.05, ** P < 0.01 compared with RD-fed mice, n = 8). (B) Hepatic *Mrp4* mRNA (left) and protein (right) levels in mice fed on RD or HFD. Immunoblot intensities for MRP4/αtubulin were quantitated. Significance was determined by Student's *t* test (* P < 0.05 compared with RD-fed mice, n = 8). (C) Effect of cAMP (20 mg/kg) injection on hepatic and serum triacylglycerol, NEFA and serum ketones levels in HFD-fed mice. Significance was determined by Student's *t* test (* P < 0.05 compared with control, n = 8). (D) Representative H&E and Oil Red O staining of liver sections from HFD-fed mice injected with cAMP (scale bar, 50 μm). (E) Effect of cAMP (20 mg/kg) injection on hepatic fatty acid oxidation gene expression. Significance was determined by Student's *t* test (* P < 0.05, ** P < 0.01 compared with control, n = 8). (F) Effect of cAMP (20 mg/kg) injection on blood glucose and insulin levels in HFD-fed mice. Significance was determined by Student's *t* test (* P < 0.05 compared with control, n = 8). (G) Effect of cAMP (20 mg/kg) injection on GTT and ITT in HFD-fed mice. Significance was determined by one-way ANOVA (* P < 0.05 compared with control mice, n = 8). (H) Schematic of proposed mechanism.

on p300, which binds to PPAR α and promotes PPAR α activity (Zhang *et al.* 2016). Thus, glucagon regulates fatty acid oxidation genes expression and PPAR α activity through multiple mechanisms.

Glucagon and its receptor contributes to the establishment of hyperglycemia and insulin resistance (Unger & Cherrington 2012, Patarrao *et al.* 2015) and glucagon receptor antagonists (Sammons & Lee 2015) as well as antisense oligonucleotides (ASOs) against glucagon receptor (van Dongen *et al.* 2014) are being assessed for the treatment of T2DM in human; however, the implication that impairment of fatty acid oxidation due to inhibition of hepatic glucagon signaling may precede, and eventually cause, hepatic steatosis suggests

limited utility for glucagon antagonism as a potential strategy for T2DM in human. Our data demonstrate that in fasted state, although glucagon action is essential for lipid metabolism, administration of extracellular cAMP may reproduce the effect of glucagon on fatty acid oxidation. Hence, the administration of cAMP in combination with glucagon receptor ASOs or antagonists may serve as a more convincing strategy in clinical use in patients with T2DM.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/JOE-16-0649>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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