Acute vs chronic exposure to high fat diet leads to distinct regulation of PKA

Edra London, Maria Nesterova and Constantine A Stratakis

Section on Endocrinology and Genetics, Program on Developmental Endocrinology and Genetics, Eunice Kennedy Shriver National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland, USA

Abstract

The cAMP-dependent protein kinase (PKA) is an essential regulator of lipid and glucose metabolism that plays a critical role in energy homeostasis. The impact of diet on PKA signaling has not been defined, although perturbations in individual PKA subunits are associated with changes in adiposity, physical activity and energy intake in mice and humans. We hypothesized that a high fat diet (HFD) would elicit peripheral and central alterations in the PKA system that would differ depending on length of exposure to HFD; these differences could protect against or promote diet-induced obesity (DIO). 12-week-old C57BL/6J mice were randomly assigned to a regular diet or HFD and weighed weekly throughout the feeding studies (4 days, 14 weeks; respectively), and during killing. PKA activity and subunit expression were measured in liver, gonadal adipose tissue (AT) and brain. Acute HFD-feeding suppressed basal hepatic PKA activity. In contrast, hepatic and hypothalamic PKA activities were significantly increased after chronic HFD-feeding. Changes in AT were more subtle, and overall, altered PKA regulation in response to chronic HFD exposure was more profound in female mice. The suppression of hepatic PKA activity after 4 day HFD-feeding was indicative of a protective peripheral effect against obesity in the context of overnutrition. In response to chronic HFD-feeding, and with the development of DIO, dysregulated hepatic and hypothalamic PKA signaling was a signature of obesity that is likely to promote further metabolic dysfunction in mice.

Introduction

High worldwide obesity rates have resulted from the combined effects of environmental and lifestyle factors including food composition and availability, portion sizes, work and commuting habits and lagging physical activity levels (Hruby & Hu 2015). The well-documented physiologic effects associated with excess adiposity include dysregulation of glucose and lipid metabolism, and altered endocrine and neuroendocrine profiles that can interfere with regulatory feedback mechanisms. Central to these negative physiologic changes is the cAMP-dependent protein kinase (PKA) pathway, an essential regulator of glucose and lipid metabolism that mediates the effects of catecholamines and other hormones such as insulin and glucagon.

The PKA holoenzyme is a tetramer consisting of 2 regulatory and 2 catalytic subunits. Four regulatory (Rⅰα, Rⅰβ, Rⅱα, Rⅱβ) and 2 catalytic (Cα, Cβ) subunit isoforms are present in human and mice that are expressed in a tissue-specific manner and have differing affinities for cAMP; α subunits are ubiquitously expressed, while β subunits have
a more restrictive expression pattern. Regulatory subunit RIA is the primary regulator of PKA in most tissues; it can compensate for disruptions in other regulatory subunits by decreasing protein turnover rate (Amieux et al. 1997, Amieux & McKnight 2002, Cazabat et al. 2014). Because of its high affinity for cAMP, increased RIA can thus lead to increased basal PKA activity. While various cellular stimuli can affect PKA RNA levels, regulation of PKA expression is ultimately thought to be controlled at the protein level.

Direct regulation of PKA signaling by nutrients or chronic overnutrition has not been thoroughly explored. Expression of PKA RIIβ and PKA activity were decreased in visceral AT from otherwise healthy obese individuals compared to lean individuals (Mantovani et al. 2009). Individuals that harbor mutations and other defects in PKA signaling molecules develop corticotropin-independent Cushing Syndrome (CS). For example, mutations of the PRKAR1A gene (that codes for Rla subunit) cause primary pigmented nodular adrenocortical disease (PNAD) (Kirschner et al. 2000, Horvath et al. 2010) and mutations of GNAS1 (that codes for the stimulatory subunit alpha or Gsα) cause primary bimorphic adrenocortical disease (Carney et al. 2011) in the context of McCune Albright syndrome. GNAS1 mutations are also infrequently found in primary macronodular adrenocortical hyperplasia (Hsiao et al. 2009). Visceral adipose tissue (AT) from patients with these forms of CS (Kirschner et al. 2000, Horvath et al. 2010) exhibited alterations in PKA expression that were associated with varying degrees of obesity (London et al. 2014b). In different settings, the other subunits may also compensate for Rla haploinsufficiency (Greene et al. 2008, Nesterova et al. 2008).

Dysregulated PKA activity in mouse models of PKA deficiency is associated with changes in adiposity, energy balance and response to obesogenic diet. The most extensively studied model is the diet-induced obesity (DIO)-resistant PKA RIIβ KO mouse that has elevated energy expenditure and basal lipolysis (Cummings et al. 1996, Planas et al. 1999) improved insulin (Schreyer et al. 2001) and leptin (Yang & McKnight 2015) sensitivities and altered locomotor activity that is sex-specific (Nolan et al. 2004). The PKA Cβ KO mouse is DIO-resistant and females display drastically reduced fat mass compared to their male counterparts, yet is phenotypically distinct from the RIIβ KO mouse (Enns et al. 2009). We recently identified DIO-resistance in the RIIα KO mouse and reported a more pronounced phenotype among females (London et al. 2014a). Interestingly, metabolic phenotypes vary considerably among RIIα KO, RIIβ KO and Cβ KO genotypes confirming specific roles and interactions for the PKA subunits in different tissues. For example, RIIβ KO and Cβ KO mice are mildly hyperphagic, unlike RIIα KO mice (Cummings et al. 1996, Enns et al. 2009, London et al. 2014a). Metabolic phenotypes of RIIβ KO and Cβ KO mice differ between sexes, and in RIIα KO mice, the observed metabolic and behavioral phenotype is more evident in female compared to male suggesting the involvement of sex hormones and/or other sex-specific factors with respect to PKA regulation of energy metabolism and homeostasis.

To test the hypothesis that chronic high fat diet (HFD) exposure would lead to dysregulation of peripheral and central PKA signaling, we gave wild-type C57Bl/6J mice ad libitum access to HFD or control (regular) diet (CD). We focused on liver and AT for our peripheral investigations because of their acute and longer-term regulatory roles in glucose and lipid metabolism and homeostasis. Further, potential feedback mechanisms linking hepatic- and adipose tissue-derived factors to CNS, and particularly hypothalamus, in the control of energy intake and appetite linked peripheral and central regulation for our purposes. As expected, long-term HFD caused increased body weight, fat mass and glucose intolerance compared to weight-matched littermates fed a CD. We found that acute HFD access led to suppressed hepatic PKA activity which is metabolically protective (London et al. 2014a). Conversely, chronic HFD access and subsequent DIO caused increased PKA activity in liver and hypothalamus that likely potentiate the deleterious physiologic effects of excess adiposity, contribute to glucose intolerance, and act as a committed step to promote development of obesity, an observation not previously made. These data suggest that there are specific interactions between diet and the PKA system even in wild-type, normal mice.

**Methods**

**Mice and diets**

Twelve-week old male and female C57Bl/6J littermates were maintained on a regular diet (control diet (CD)) from weaning at approximately 21 days. At 11–12 weeks of age, mice were randomly assigned to either the CD or HFD group and provided ad libitum access to water and their respective diet for 14 weeks (n=7–9/group per sex) or 4 days (n=6–8/group). Same-sex mice were group housed within treatment group 2–4 mice per cage and maintained on a 12-h light:darkness cycle at 22–23°C. Mice were
originally obtained from Jackson Labs and were then inbred and maintained at the NIH 10A animal facility for more than 5 years prior to these studies.

The CD, NIH-31 Open Formula (Harlan Teklad, Indianapolis, IN, USA), has an energy density of 3.0 kcal/g with 24, 14 and 62% of the total energy derived from protein, fat and carbohydrate. The HFD used was high fat soft pellet chow (F3282; Bio-Serv, Frenchtown, NJ, USA) that has an energy density of 5.5 kcal/g; 21, 36 and 36% of energy was derived from protein, fat and carbohydrate.

**Weight gain, adiposity and glucose tolerance**

Mice were weighed at baseline and then weekly at 09:00h during the experimental period and at time of killing. Body composition was measured at the conclusion of the 14-week study by EchoMRI-100H body composition analyzer for mice (EchoMRI, Houston, TX, USA). Intraperitoneal glucose tolerance test was performed at week 14 of the long-term diet study. Mice were overnight fasted (16 h) prior to IP glucose injection (2 g/kg) (Sigma Aldrich). Blood glucose was measured in 3 µL of tail blood at baseline, 15, 30, 60 and 120 min with a Contour glucometer and test strips (Bayer Healthcare LLC; Tarrytown, NY, USA).

**Tissue and serum collection**

Mice were killed by slow replacement of air with CO₂ followed by cervical dislocation and exsanguination. Dissected tissues were snap frozen in liquid nitrogen and stored (−80°C). Tissues aliquoted for gene expression studies were first preserved in RNAlater (Qiagen). For immunofluorescence experiments, mice were killed and immediately perfused with ice-cold 4% PFA and then post-fixed for 24 h in 4% PFA and 4°C for 10 min. Total protein concentrations were determined by BCA assay (Pierce). PKA enzymatic activity was measured using a previously described method that employs P<sup>32</sup>-labeled ATP and kemptide substrate with 1 mM EDTA, 1 mM dithriothreitol, and protease inhibitor cocktail I (EMD Biosciences). Hypothalamus was dissected from whole brain under a dissecting microscope (Nikon Advance) in ice-cold buffer (10 mM Tris–HCl (pH 7.5), 1 mM EDTA, 1 mM dithriothreitol, and protease inhibitor cocktail I (EMD Biosciences). Hypothalamus was dissected from whole brain under a dissecting microscope (Nikon SMZ 1500). Cell lysates were centrifuged at 10,000g and 4°C for 10min. Total protein concentrations were determined by BCA assay (Pierce). PKA enzymatic activity was measured using a previously described method that employs P<sup>32</sup>-labeled ATP and kemptide substrate with

**Quantitative reverse transcription-PCR assay**

Total RNA was extracted using RNEasy Mini and Lipid Mini kits (Qiagen) for liver and AT, respectively, and included on-column DNase digestion with DNase I (Invitrogen). cDNA was synthesized from 250 ng (AT) and 500 ng (liver) total RNA with SuperScript III First Strand Synthesis Supermix for qRT-PCR (Invitrogen). Relative mRNA expression was quantified by qRT-PCR (VIAA7; Applied Biosystems) by calculating the delta CT and determining fold-change as the 2<sup>−ΔΔCT</sup> value (Livak & Schmittgen 2001). Previously tested and optimized qPCR primers were used (Supplementary Table 1, see section on supplementary data given at the end of this article). Actb1 was the housekeeper gene for liver and the averaged value for Actb1, Rplp0, B2m and Hprt was used for AT. PCR products run on 2% agarose gels confirmed the presence of a single band at the expected amplicon size as an additional quality control check subsequent to melt curve analysis.

**Western blot**

AT, liver and brain tissues were homogenized in ice-cold TPER lysis buffer (Thermo Fisher Scientific) with 1:100 protease and phosphatase inhibitor cocktails (EMD Biosciences, La Jolla, CA, USA) using a BulletBlender and RNase-free beads (Next Advance, Averill Park, NY, USA). Total protein was quantified by BCA assay (Pierce). Western blots were performed by loading 15–30 µg total protein per well (depending on tissue type) and probing with commercially available antibodies (Supplementary Table 2). Membranes were visualized on a ChemiDoc imaging system and densitometry analysis was performed to determine relative expression (ImageLab 4.0, BioRad).

**Immunohistochemistry**

Slides prepared from 20 µm thick coronal brain sections were probed with phosphorylated CREB (pCREB) (s133) (Cell Signaling) and then Alexa Fluor 594 secondary antibody (Santa Cruz Biotechnology), and counterstained with Dapi nuclear stain (Thermo Fisher Scientific). Microphotographs of hypothalamus were taken using uniform exposure settings for all sections with an Axioplan microscope and analyzed using Zen2012 software (Zeiss).

**PKA activity**

Liver, AT and brain samples were homogenized with a Bullet Blender Tissue Homogenizer and lysis beads (Next Advance) in ice-cold buffer (10 mM Tris–HCl (pH 7.5), 1 mM EDTA, 1 mM dithriothreitol, and protease inhibitor cocktail I (EMD Biosciences). Hypothalamus was dissected from whole brain under a dissecting microscope (Nikon SMZ 1500). Cell lysates were centrifuged at 10,000g and 4°C for 10 min. Total protein concentrations were determined by BCA assay (Pierce). PKA enzymatic activity was measured using a previously described method that employs P<sup>32</sup>-labeled ATP and kemptide substrate with
or without added cAMP (5 µM) (Nesterova et al. 1996). Calculated PKA/kinase activities were normalized to total protein concentration.

**Statistical analysis**

Data were described using simple descriptive statistics, which are reported as mean ± standard error of the mean (s.e.m.); relative changes are described as percent of each respective comparison. All data distributions were assessed for approximate normality with the Shapiro–Wilk test. Continuous data (mRNA and protein expression, PKA activity, BW, IPGTT) were compared between two independent groups using t-tests. A P value less than 0.05 was considered statistically significant. Data were analyzed using SAS 9.3 (SAS Institute, Inc.; Cary, NC, USA) and graphed with GraphPad Prism 7.

All mouse procedures were approved by and conducted in accordance with the Eunice Kennedy Shriver National Institute for Child Health and Human Development Institutional Animal Care and Use Committee.

**Results**

**Chronic HFD-feeding produced expected increases in body weight, fat mass and blood glucose levels in mice**

Chronic HFD-feeding significantly increased weight gain in mice (Supplementary Fig. 1A and B). Female and male mice weighed 65% and 53% more after 14-week ad lib HFD access compared to CD-fed mice, respectively. HFD access for 4 days did not cause significant weight gain in female or male mice. 14-week HFD-feeding caused more than 4-fold and 3-fold increases in total fat mass in female and male mice, respectively (Supplementary Fig. 1C and D).

After 14 weeks of HFD access, non-fasted blood glucose was higher in male and female mice (P<0.05) compared to CD-fed littermates (data not shown). Glucose tolerance, tested after 14-week HFD-feeding...
was significantly impaired in both sexes (Supplementary Fig. 2A and B).

**Hepatic PKA activity and expression are differentially regulated by acute and chronic HFD-feeding**

We first examined the effects of chronic HFD-feeding on the regulation of PKA enzymatic activity and subunit expression and observed a more profound effect in female mice (Supplementary Fig. 3 for male mouse data). We therefore carried out the subsequent analyses in tissues from female mice.

Acute (4 day) access to HFD tended to cause a decrease in basal hepatic PKA activity (Fig. 1A) \( (P=0.06) \), and significantly decreased hepatic phosphorylated cAMP response element binding protein (pCREB) (S133) levels (Fig. 1B) \( (P<0.03) \). In liver, acute HFD-feeding did not alter the regulation of PKA subunits at the mRNA level (Fig. 1C), but protein expression of the major PKA catalytic subunit, \( \alpha \), was decreased (Fig. 1D) \( (P<0.05) \).

Chronic (14 week) HFD-feeding caused a significant increase in cAMP-stimulated hepatic PKA activity \( (P<0.02) \) (Fig. 1E). Phosphorylated CREB levels were higher in liver of mice after chronic HFD-feeding (Fig. 1F) \( (P<0.03) \), and overall CREB expression appeared to be upregulated by chronic HFD. Chronic HFD-feeding led to the overall downregulation of PKA subunit mRNA (1G). mRNA levels of \( Prkar1a \), \( Prkar2a \), \( Prkaca \) and \( Prkacb \) were suppressed by 36\% \( (P<0.003) \), 60\% \( (P<0.003) \), 32\% \( (P<0.006) \) and 32\% \( (P<0.03) \) compared to levels of the control group (Fig. 1G). In line with the observed increase in hepatic PKA enzymatic activity, PKA catalytic subunits \( \alpha \) and \( \beta \) protein expression was increased in HFD-fed mice (Fig. 1H) \( (P<0.05) \). The discrepancy between RNA and protein was not surprising as PKA subunit expression has been shown to be regulated primarily by changes in protein stability (McKnight et al. 1998).

To confirm the downstream effects of the changes observed in hepatic PKA activity we examined expression and phosphorylation status of downstream PKA targets with important roles in regulating energy homeostasis. After 4 day HFD-feeding, expression of phosphoenolpyruvate carboxykinase (PEPCK) was lower than that of CD-fed mice, while chronic HFD exposure caused increased PEPCK expression (Fig. 2A). After acute 4 day HFD, phosphorylated acetyl co-A carboxylase (pACC) levels did not differ from control levels, but total ACC expression tended to be higher in the 4 day HFD mice (Fig. 2B). pACC was significantly higher after chronic HFD-feeding (Fig. 2B); total ACC levels also appeared to be increased. In an attempt to understand
the mechanism behind decreased basal hepatic PKA activity and concurrent enhancement of ACC, we examined levels of phosphorylated AMPK at tyrosine 172 (T-172) levels after 4 day HFD (Fig. 2C). pAMPK (T-172) levels tended to be increased after acute exposure to HFD but were not significantly higher (Fig. 2C).

**Chronic HFD-feeding increases hypothalamic PKA activity and alters cAMP signaling in response to fasted and fed states**

Acute HFD exposure did not affect basal or cAMP-induced PKA activities in hypothalamus (Fig. 3A), nor did it impact protein expression of either PKA catalytic subunit α or β (Fig. 3B). Conversely, chronic HFD-feeding increased basal and total hypothalamic PKA activities of approximately 20-fold and 4-fold, respectively, compared to levels in control-fed mice (Fig. 3C). Hypothalamic expression of the predominant neuronal PKA catalytic subunit, Cβ was increased after chronic HFD and levels of Cα protein also tended to be higher in HFD-fed mice (Fig. 3D). Fed-state PKA enzymatic activity was also assayed in hippocampus and striatum after long-term HFD exposure, but no differences were observed in mice (data not shown).

To better understand the changes in PKA signaling that accompany chronic HFD-feeding we investigated CREB phosphorylation in arcuate nucleus (Arc), dorsomedial hypothalamus (DMH) and ventromedial hypothalamus (VMH) in both fed and overnight fasted mice (Fig. 4A, B and C). After 16 h overnight fast, both CD- and HFD-fed mice displayed intense staining for pCREB in Arc (Fig. 4A and C, left panels). DMH was also positive for pCREB, but staining intensity was greater in CD-fed mice (Fig. 4B, left panels). Staining for pCREB in VMH was more diffuse than in the other nuclei examined and did not seem to differ among treatment groups (Fig. 4A). After chronic HFD access, fed-state mice exhibited intense pCREB staining in Arc similar to what was observed in fasted mice, whereas non-fasted control mice did not (Fig. 4A and C).

**Chronic HFD-feeding alters PKA subunit expression in AT, yet produces only subtle changes in PKA activity**

Acute exposure to HFD did not elicit significant changes in regulation of the PKA pathway in AT as evidenced by the lack of difference in basal and cAMP-induced PKA activities between CD and HFD-fed mice (Fig. 5A). Protein expression of PKA Cα was unchanged and while RIIβ
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tended to be higher they were not significantly increased after acute HFD exposure (Fig. 5B).

While chronic HFD exposure did not initiate significant changes in either basal or cAMP-induced PKA activities in AT (Fig. 5C), the ratio of basal to total PKA activity was decreased in HFD-fed mice (Fig. 5D, P < 0.03). In AT, RIIβ protein was significantly higher after chronic HFD, and while Ca protein tended to be higher the difference was not significant (Fig. 5E). Despite the lack of profound changes in PKA activity after chronic HFD, mRNA expression levels of all the PKA subunits except Ca were significantly increased in AT compared to CD-fed mice suggesting alterations in regulation of the PKA system (Fig. 5F).

Discussion

Chronic intake of a high fat, high calorie diet has been well documented for its ability to induce weight gain and metabolic dysfunction in humans and animals alike. Inter-individual variability in the susceptibility or resistance to

Figure 4
Phosphorylation of CREB (S133) varies in Arc, VMH and DMH in fasted vs fed-state and is dysregulated after chronic HFD-feeding in mice. (A) Representative immunofluorescent staining for pCREB (S133) (Alexa fluor 594), counterstained with DAPI in hypothalami of 16 h-fasted and non-fasted mice that were chronically exposed to either HFD or CD; scale bars = 200 µm. (B) Representative immunofluorescent staining for pCREB (S133) (Alexa fluor 594) in DMH; scale bars = 100 µm. (C) Representative immunofluorescent staining for pCREB in Arc (S133) (Alexa fluor 594); scale bars = 100 µm. Immunofluorescence experiments were repeated in 3 cohorts of mice totaling 4–6 mice per treatment group. Arc, arcuate nucleus; VMH, ventromedial hypothalamus; DMH, dorsomedial hypothalamus.
DIO, however, is not well understood. Genetic background and environment are contributors, but gaining a clearer picture of the physiological underpinnings of this variability could help develop viable treatment plans to combat the obesity epidemic. For this reason we sought to explore how chronic exposure to an obesogenic diet impacts one of the primary signaling pathways that regulates energy metabolism, the PKA pathway. In order to model the most common form of human obesity, non-monogenic obesity caused by chronic consumption of a Western diet we used a wild-type mouse model of DIO and examined regulation of the PKA system at two distinct time points.

Nutrient sensing in liver regulates peripheral and central energy homeostasis by maintaining appropriate responses in glucose and lipid metabolisms during both fed and fasted states. In recent decades, what has been termed a ‘Western diet’ has proven capable of upsetting normal hepatic function thus leading to dysregulated energy metabolism. HFDs irrespective of fatty acid composition can cause increased fat storage in liver, elevated circulating triglycerides ([de Meijer et al. 2010]) and decreased insulin sensitivity ([Samuel et al. 2004]). Diets high in sucrose and fructose, prevalent in contemporary society, can acutely increase circulating triglycerides ([London & Castonguay 2011]) and humans ([Cybulska & Naruszewicz 1982]). High intake of fructose-containing sweeteners can also increase hepatic triglyceride levels ([Lowndes et al. 2014]) and decrease insulin sensitivity ([Beck-Nielsen et al. 1980]) in human subjects. These examples demonstrate

**Figure 5**

Chronic HFD-feeding impacts PKA expression in gonadal AT while PKA signaling is only modestly altered. (A) Basal and cAMP-stimulated (5 µM cAMP) PKA activities after acute 4 day CD- or HFD-feeding, n=6–8/group. (B) Representative Western blots and quantification of PKA Cα and RIIβ in gonadal AT of mice after 4 day CD- or HFD-feeding, n=6–8/group. (C) Basal and cAMP-stimulated (5 µM cAMP) PKA activities after chronic 14 week CD- or HFD-feeding and ratio of basal to cAMP-stimulated PKA activities, n=6–8/group. (D) Representative Western blots and quantification of PKA Cα and RIIβ in AT of mice after 14 week CD- or HFD-feeding, n=6–8/group. (E) mRNA expression of PKA subunits in AT after 14 week HFD-feeding (n=8/group).

All values are mean ± s.e.m.; *P<0.05. PKA enzymatic activity assays were repeated twice and Western blotting was repeated three or more times.
how diet composition impacts key signaling pathways both acutely and chronically and can lead to metabolic dysregulation, yet the point at which this dysregulation becomes increasingly difficult to reverse is unclear.

Mounting evidence shows that inhibition of hepatic PKA activity or its downstream targets is therapeutically beneficial in the treatment of type II diabetes-related hyperglycemia and complications related to hyperglycemia. It was recently shown that the mechanism by which biguanides, the most commonly prescribed class of T2DM drugs attenuate hepatic glucose output is by the reduction of hepatic cAMP and the subsequent decreases in PKA activation and phosphorylation of downstream targets (Miller et al. 2013). We reported decreased hepatic PKA activity in the PKA RIIaKO mouse that conferred protection from diet-induced fatty liver and glucose intolerance (London et al. 2014a). Furthermore, hyperglycemic and hyperglucagonemic db/db mice in the fasted state had enhanced hepatic phosphorylation of CREB and IRE1α, a PKA phosphorylation target identified as a downstream regulator of glucose metabolism (Mao et al. 2011). Cumulatively, these data support the therapeutic usefulness of inhibiting cAMP/PKA signaling at various points in the pathway.

Here we provide direct evidence that altered PKA expression in DIO and hepatic PKA hyperactivity is a characteristic of dietary obesity that may be part of a ‘committed step’ that can be difficult to reverse in DIO. Chronic HFD-feeding causes increased hepatic protein expression of both PKA catalytic subunits, Ca and Cβ. This increase in catalytic subunit expression explains our finding of elevated cAMP-stimulated PKA activity and supports the observed tendency toward increased basal PKA activity.

The increased expression of PKA catalytic subunit protein was likely due to increased protein stability. This observation could explain the concurrent downregulation of the PKA subunits at the transcriptional level that are part of a feedback loop, and further exemplify the dysregulation of hepatic PKA in DIO. Changes in hepatic CREB phosphorylation mimicked the decrease and increase in PKA activity in acute and chronic HFD exposure, respectively, and suggest differential gene regulation by CREB in response to these two conditions.

The metabolic effects of altered hepatic PKA regulation after both acute and chronic HFD were investigated by examining downstream PKA targets, PEPCK, ACC and AMPK (Fig. 2). PEPCK, the rate-limiting step in gluconeogenesis has been shown to be increased in obesity in mice (Triscari et al. 1979) and humans alike (Oberkofler et al. 2010). We replicate the previously described phenomenon of elevated PEPCK in obesity and show that it is associated with increased hepative PKA activity. Additionally, we show that acute HFD exposure leads to decreased PEPCK expression in mice that is associated with decreased basal hepatic PKA activity and appears to be metabolically protective in this early stage of caloric excess. The regulatory effects on ACC and pACC were less clear. While pACC is increased in DIO as expected (Fig. 2B), the lack of change pACC after acute HFD exposure may suggest that the tendency toward decreased basal PKA activity in liver was able to attenuate phosphorylation of ACC. However, when we quantified total ACC levels in the same mice, they tended to be higher. We examined AMPK levels since pAMPK is also a regulator of ACC (Witters et al. 1991) and phosphorylation of threonine 172 exerts an inhibitory effect on PKA, an effect that was observed after metformin administration (Aw et al. 2014). Similarly pAMPK (T-172) levels tended to be but were not significantly higher in 4 day HFD-fed mice (Fig. 2C). This seemingly protective response in the face of short-term excess nutrition is likely part of a previously reported compensatory response that can counter the initial increased flux through intermediary metabolic pathways in favor of energy balance. In adult non-diabetic men energy expenditure and carbohydrate oxidation increased in response to acute hypercaloric Western diet (Larson et al. 1995), and similarly in mice, energy expenditure increased initially upon initiation of HFD-feeding but this effect was absent by the end of week one (So et al. 2011). Whether the dysregulation we observed is directly induced by chronic HFD-feeding or is secondary to hepatic inflammation and increased adiposity is unclear.

The central defects we observed in PKA signaling appear to be secondary to increased adiposity and altered peripheral inputs since acute HFD-feeding did not elicit changes. Both basal and cAMP-stimulated PKA activity increased in hypothalamus as a result of chronic HFD-feeding, but hippocampus and striatum were not significantly affected. Visualizing hypothalamic CREB phosphorylation in fasted and fed mice highlighted how chronic HFD can impact PKA signaling in distinct nuclei (Fig. 4).

In Arc, fasting induced CREB phosphorylation among CD and HFD-fed mice, although staining for pCREB appeared denser in the HFD-fed mice (Fig. 4C). Interestingly, pCREB staining was barely detectable in Arc of non-fasted control mice; however, staining for pCREB in Arc of non-fasted HFD-fed mice mimicked what was
seen in fasted mice (Fig. 4C). We hypothesize that chronic hyperleptinemia, hyperinsulinemia and perhaps changes in other hormones characteristic of DIO, contribute to the increased basal and cAMP-induced hypothalamic PKA signaling and are involved in deregulation of the PKA system in hypothalamus. Yang and McKnight reported that decreased PKA signaling in Arc and decreased hypothalamic CREB phosphorylation was associated with leptin hypersensitivity in Prkar2b KO mice (Yang & McKnight 2015). This altered PKA signaling was linked to decreased energy intake and weight gain in fasted Prkar2b KO mice that were administered leptin. Our data add to this by confirming enhanced CREB phosphorylation (S133) in Arc in the fasted state and also in Arc of non-fasted DIO mice, a pattern not observed in non-fasted lean mice. We postulate that CREB activation in Arc in fed DIO mice could play a role in the dysregulation of hypothalamic satiety signaling and the decreased inhibition in the presence of palatable food.

Altered CREB phosphorylation was observed in other hypothalamic nuclei implicated in regulation of hunger, satiety and feeding behavior. pCREB staining was noticeably present in VMH of fasted CD-fed mice and present to a lesser extent in fed CD mice, yet pCREB staining intensity in VMH was similar in HFD-fed mice regardless of their feeding status (Fig. 4A). In DMH of CD-fed mice, staining for pCREB was more intense in fasted compared to fed mice, and pCREB staining was notably less intense in DIO mice (Fig. 4B). DMH involvement in food-entrainment and reported increases in DMH activation at mealtime are involved in anticipatory behaviors including bouts of physical activity (Gooley et al. 2009). The clear differences in hypothalamic CREB activation among CD- and HFD-fed mice identified nuclei where PKA-mediated dysregulation can impact circuits controlling energy intake behaviors.

While there were clear changes in liver and hypothalamus of mice with established DIO, differences in AT were more subtle. In obese human subjects decreased expression of the PKA RIβ as well as R1α and RIα was observed in obese compared to lean subjects (Mantovani et al. 2009). However, deficiency of RIβ in mice enhanced basal AT PKA activity and lipolysis, and decreased fat mass (Cummings et al. 1996). While enhanced basal lipolysis and altered PKA regulation in AT are characteristics of RIβKO mice, the changes observed in AT alone do not confer the lean phenotype. RIαKO mice also resist DIO independent of changes in AT (London et al. 2014a). The lack of altered regulation of PKA activity in AT of DIO mice provides additional evidence that dysregulation of PKA activity in AT may not be a primary component of PKA's role in obesity. We initially expected to find decreased basal and perhaps cAMP-stimulated PKA activity that could promote increased fat mass in DIO mice. Despite increased RIIβ and the tendency toward increased Caα protein in DIO mice, neither basal nor total PKA activities differed significantly from lean mice. We did, however, find that the ratio of basal to total PKA activity in DIO mice was reduced suggesting a change in the overall profile of PKA activity in DIO. This is in agreement with the idea that decreased basal PKA activity in AT contributes to increased lipid storage and total adiposity.

We identified sex-specific differences with regard to PKA regulation of energy balance in DIO that is seen in mice with PKA deficiencies that exhibit various sexual dimorphisms in metabolic phenotypes (Cummings et al. 1996, Enns et al. 2009, London et al. 2014a). Changes in PKA expression were more pronounced in female compared to male mice. The 3 times greater prevalence of CS in females lends further support for sex-specific metabolic regulation by PKA as CS is associated with defects in a number of molecules integral to the PKA pathway (Carney et al. 2015). Sexual dimorphism in AT function and distribution has been documented; for a thorough overview of the literature see the review by Palmer and Clegg (Palmer & Clegg 2015). Our data in a mouse model implicate PKA regulation in the development of DIO that is present in both sexes, but more distinct in females.

In summary, there is differential regulation of the PKA signaling pathway in response to acute and chronic consumption of an obesogenic diet in a mouse model of DIO. Acute intake of excess calories from a high sugar, HFD produced an initial protective effect on hepatic PKA regulation by compensated for excess substrate. In the case of chronic HFD intake the PKA system became dysregulated both centrally and peripherally. It is, however, not possible to delineate whether the PKA signaling pathway is regulated directly by changes in flux of intermediary metabolism substrates, adipokines or inflammatory response. Further study of PKA dysregulation over time during HFD access, may aid in establishing a tipping point in the development of DIO with respect to the PKA system.

Supplementary data
This is linked to the online version of the paper at http://dx.doi.org/10.1530/JME-16-0188.
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.

Funding
This work was supported by NICHD, NIH intramural research program project Z01-HD-000642-04 to Dr C A Stratagakis.

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Received in final form 21 March 2017
Accepted 18 April 2017
Accepted Preprint published online 18 April 2017