Homocysteine suppresses lipolysis in adipocytes by activating the AMPK pathway

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Homocysteine suppresses lipolysis in adipocytes by activating the AMPK pathway. Am J Physiol Endocrinol Metab 301: E703–E712, 2011. First published July 12, 2011; doi:10.1152/ajpendo.00050.2011.—Hyperhomocysteinemia (HHcy) is an independent risk factor for coronary artery disease. Emerging evidence suggests that HHcy is also associated with adipocyte tissue dysfunction. One of the principal functions of adipose tissue is to provide energy substrate via lipolysis. In the present study, we investigated the effects of homocysteine (Hcy) on lipolysis in adipocytes. We found that Hcy inhibited release of glycerol and fatty acids, two typical indicators of the lipolytic response, in primary adipocytes and fully differentiated 3T3-L1 adipocytes in a dose-dependent manner under both basal and isoproterenol-stimulated conditions. In differentiated 3T3-L1 adipocytes, decreased glycerol and free fatty acid (FFA) release was associated with elevation of intracellular TG content. Further studies showed that Hcy-mediated antilipolytic responses were independent of the cyclic AMP-PKA and MEK-ERK1/2 pathways. However, Hcy increased phosphorylation levels of AMP-activated protein kinase (AMPK) and its downstream enzyme acetyl-CoA carboxylase. Compound C, an AMPK inhibitor, abolished Hcy-induced reduction of glycerol and FFA release under both basal and isoproterenol-stimulated conditions. Furthermore, AMPKα1 siRNA reversed Hcy-inhibited glycerol release. Supplementation of exogenous Hcy in the diet for 2 wk lowered circulating glycerol and FFA levels. Moreover, Hcy supplementation was associated with elevated levatin levels and reduced adiponectin levels in plasma. These results show that Hcy inhibits lipolysis through a pathway that involves AMPK activation.

ADIPOSE TISSUE IS A KEY PLAYER in whole body energy regulation. Triglycerides (TG), the main form of energy storage, are synthesized and stored in the cytosolic lipid droplets of adipocytes during times of energy excess and are mobilized via lipolysis, in which the hydrolysis of TG releases free fatty acids (FFAs) and glycerol for energy requirement of other organs. As such, the pathways controlling intracellular TG storage and lipolysis in adipocytes are tightly regulated. Many tissues are capable of synthesizing TG; however, lipolysis for release of FFAs as energy supply for other tissues is unique to adipocytes. Dysregulated adipose tissue TG metabolism has profound effects on whole body energy homeostasis. Although an imbalance between energy intake and expenditure is undoubtedly a major determinant in the development of fat mass, altered lipolysis could also be an element leading to obesity. Several gene polymorphisms associated with altered adipocyte lipolytic function are associated with obesity (17, 18, 24). Conversely, excessive lipolytic rates, together with impairment in FFA utilization by other organs, may be a major contributor to the metabolic abnormalities associated with obesity and lead to type 2 diabetes (2).

Lipolysis is under strong hormonal regulation, and intracellular lipolytic systems have been well characterized (9, 14). Catecholamines are major hormones that markedly stimulate lipolysis through elevating intracellular cyclic AMP (cAMP) production and subsequently activating cAMP-dependent protein kinase A (PKA). PKA then phosphorylates hormone-sensitive lipase (HSL) in adipocytes and causes its translocation from the cytosol to the lipid droplet surface, a crucial step for the lipase to access its triacylglycerol substrates (9). Alternatively, catecholamines also induce the MEK-ERK1/2 pathway activation in adipocytes, which is another important signaling pathway that modulates lipolysis (14). In addition to these conventional pathways, several recent studies have demonstrated that activation of AMP-activated protein kinase (AMPK), a key sensor of cellular energy level, suppresses HSL phosphorylation/activation and exerts antilipolytic effects in adipocytes (1, 11).

Homocysteine (Hcy) is a sulfur-containing amino acid formed during the metabolism of methionine. Abnormal methionine/Hcy metabolism is associated with a variety of pathological states (5, 22, 23, 28). The association of altered Hcy metabolism and both coronary and peripheral atherothrombosis has been well documented, and hyperhomocysteinemia (HHcy) has been implicated as an independent risk factor for coronary heart disease (5). Moreover, increased plasma and liver Hcy levels have been associated with chronic alcohol exposure in both experimental animals and humans and postulated to play a pathological role in the development of alcoholic liver disease (22, 28). Although some epidemiological literature suggests that HHcy is associated with adipose tissue dysfunction (10, 23, 25), direct evidence has emerged only recently. We reported initially that chronic alcohol exposure increases Hcy concentration in adipose tissue, which contributes to reduced adiponectin production in alcohol-fed mice (28). Moreover, we showed that supplementation of betaine, a Hcy-reducing agent, improves adipose tissue func-
tions in mice challenged with high-fat diets (33). A recent study by Li et al. (20) demonstrated that HHCy induced by dietary Hcy supplementation increases insulin resistance by upregulating resistin production from adipocytes in vivo and in vitro. Thus, available evidence indicates that abnormal Hcy metabolism is associated with malfunction of adipose tissue. Based on these recent findings, we conducted both in vivo and in vitro experiments to examine the effect of Hcy in the lipolytic response of adipocytes, and we report that Hcy suppresses the lipolysis process via activation of the AMPK pathway.

MATERIALS AND METHODS

Cell culture and induction of differentiation in 3T3-L1 cells. Mouse embryonic fibroblasts 3T3-L1 cells were obtained from American Type Culture Collection (Manassas, VA) and grown in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% antibiotics (Cellgro, Manassas, VA) until confluence and induced to differentiate. Briefly, 2 days postconfluence (day 0), cells were exposed to differentiation medium containing 0.5 mM isobutylmethylxanthine, 1 µM dexamethasone, and 1.67 µM insulin (Sigma, St. Louis, MO), and 10% FBS for 3 days. Cells were then transferred to DMEM with 1.67 µM insulin and 10% FBS and refed every 2 days. Maturation of adipocytes was confirmed by Oil Red O staining of lipid droplets on day 7.

Isolation and culture of primary adipocytes. Male C57BL/6 mice (8–9 wk old) were used to obtain primary adipocytes. Briefly, mice were anesthetized and euthanized via cervical dislocation. Epididymal fat pads were harvested, washed in phosphate-buffered saline (PBS; pH 7.4) at room temperature, and minced thoroughly (2–3 mm) in collagenase solution (0.2 mg/ml collagenase A, 4 ml/g adipose tissue). This mixture was incubated at 37°C, with shaking at 120 rpm for 30 min. After digestion, the mixture was filtered through a 250-µm gauze mesh into a 50-mL conical polypropylene tube and allowed to stand for 2–3 min. The floating layer of adipocytes was washed three times and incubated at 37°C in DMEM containing 1% bovine serum albumin.

Oil Red O staining and quantification of lipid accumulation in adipocytes. Lipid droplets in mature adipocytes were stained with Oil Red O. Cells were fixed with 10% formalin and incubated with Oil Red O (Sigma) in 100% isopropanol for 1 h at 60°C. Cells were then washed twice with distilled water to remove excess dye and photographed under microscopy. To quantify intracellular lipid accumulation, stained lipid droplets were dissolved with 100% isopropanol for 10 min. Optical density was measured at 500 nm by spectrophotometer.

Glycerol assay. Glycerol content in the culture medium of adipocytes served as an index of lipolysis and was determined at absorption at 540 nm by use of a colorimetric assay kit (Cayman Chemical, Ann Arbor, MI). Glycerol concentrations were expressed as micromoles per milliliter.

FFA assay. The concentration of FFAs in the culture medium was determined by colorimetric assay (BioVision, Mountain View, CA). Briefly, 50 µl of culture medium was mixed with 2 µl of a FFA probe and 2 µl of enzyme mixture, and the reaction was developed for 30 min at 37°C. Absorbance at 570 nm was spectrophotometrically measured in a 96-well plate. FFA concentrations were expressed as micromoles per milliliter.

cAMP determination. Differentiated 3T3-L1 adipocytes were exposed to indicated treatments for 15 min after Hcy (0.1 or 0.5 mM) pretreatment for 2 h. Adipocytes were then washed with PBS and lysed in 0.1 mM hydrochloric acid. After centrifuging at 600 g at room temperature, supernatants were used to measure cAMP contents with a cAMP assay kit (BioVision). We used the rest of the adipocytes in each group to quantify protein content by the Bradford method for equal loading among different groups.

ELISA for AMPK activity. After 24-h incubation with insulin-free medium, differentiated 3T3-L1 adipocytes were exposed to Hcy at indicated doses for 2 h. Adipocytes were directly lysed in extraction buffer (50 mM Tris-HCl, pH 7.5, 50 mM NaF, 5 mM sodium pyrophosphate, 1 mM EDTA, 10% glycerol, 1 mM dithiothreitol, 1% Triton X-100), and the extract was centrifuged at 16,000 g for 10 min. The resulting supernatant was collected to determine intracellular AMPK activity via a Cyclex AMPK Kinase Assay ELISA kit (Cyclex, Nagano, Japan) according to the manufacturer’s instructions.

Suppression of AMPK expression by small interfering RNA. Stealth RNAi small interfering RNA (siRNA) targeting the mouse AMPKα1 gene and a control siRNA containing a scrambled sequence (Invitrogen, Carlsbad, CA) were transfected by Lipofectamine RNAiMAX Transfection Agent (Invitrogen) according to the manufacturer’s instructions.

Western blotting. Fully differentiated 3T3-L1 or primary adipocytes were lysed in RIPA buffer, and isolated proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to 0.45 µm of polyvinylidene difluoride membrane. After transfer, membranes were blocked in 5% bovine serum albumin in PBS with 0.1% Tween 20 and probed with anti-phospho-AMPKα, anti-AMPKα, anti-phospho-acetyl-CoA carboxylase (ACC), anti-phospho-ERK1/2, or anti-ERK1/2 antibodies (Cell Signaling Technology, Danvers, MA). Horseradish peroxidase-conjugated secondary antibodies and an enhanced chemiluminescence substrate kit were used in the detection of specific proteins.

Quantitative real-time RT-PCR. Total RNA, from either 3T3-L1 adipocytes or adipose tissue, was isolated with a phenol-chloroform extraction. For each sample, 1.0 µg of total RNA was reverse transcribed using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). The cDNA was amplified in MicroAmp Optical 96-well reaction plates with a SYBR Green PCR Master Mix (Applied Biosystems) on an Applied Biosystems Prism 7000 sequence detection system. Relative gene expression was calculated after normalization by a housekeeping gene (mouse or human 18S rRNA).

Animal care and feeding. Male C57BL/6 mice (8 wk old; Charles River Laboratories, Wilmington, MA) weighing 25 ± 0.5 g were housed in the Biologic Resources Laboratory at the University of Illinois at Chicago. Studies were approved by the Institutional Animal Care and Use Committee, which is certified by the American Association for Accreditation of Laboratory Animal Care. Ten mice were randomly assigned into two groups and fed liquid diets containing (%energy intake) 18% protein, 35% fat, and 47% carbohydrate without control or with supplementation of 0.1% Hcy for 2 wk. Food intake and body weight were recorded daily and weekly, respectively. At the end of the experiment, mice were euthanized and plasma and adipose tissue samples harvested.

Plasma biochemical assays. Plasma biochemical assays were performed with the following commercially available kits: TG and cholesterol (Fisher Diagnostics, Middletown, VA), glycerol (Cayman Chemical), FFAs (BioVision), and leptin and adiponectin (Linco Research, St. Charles, MO).

Statistical analysis. Data are expressed as means ± SD. Statistical analysis was performed using a one-way ANOVA and was analyzed further by Newman-Keuls test for statistical difference. Differences between treatments were considered to be statistically significant at P < 0.05.

RESULTS

Homocysteine inhibits lipolysis in fully differentiated 3T3-L1 and primary adipocytes. Hydrolysis of TG by cellular lipases results in the release of glycerol and FFAs from adipocytes. To determine the effect of Hcy on lipolysis under basal conditions, both fully differentiated 3T3-L1 and primary adipocytes were treated with DL-Hcy (0.1 and 0.5 mM) for 24
Homocysteine increases TG contents in 3T3-L1 adipocytes. To further examine the antilipolytic effect of Hcy, we measured intracellular TG content in 3T3-L1 adipocytes by Oil Red O staining and biochemical assay. Compared with untreated cells, inclusion of Hcy in the culture medium for 3 days increased intracellular TG accumulation (Fig. 2) in a dose-dependent manner.

cAMP-PKA and ERK1/2 pathways are not involved in the antilipolytic effect of Hcy in 3T3-L1 adipocytes. Intracellular cAMP elevation and ERK1/2 pathway activation are well-documented pathways implicated in the lipolytic response in adipocytes (14, 28). To test whether these pathways are mechanistically involved in Hcy-induced inhibition on lipolysis, we first examined the effect of Hcy on intracellular cAMP level in 3T3-L1 adipocytes. As shown in Fig. 3A, Hcy exposure to Hcy did not significantly increase cAMP levels under either basal or isoproterenol-stimulated conditions, although isoproterenol stimulation markedly elevated intracellular cAMP levels compared with untreated cells. Similarly, Hcy failed to alter ERK1/2 phosphorylation and expression levels under both basal and isoproterenol-stimulated conditions (Fig. 3, B and C).

Homocysteine activates the AMPK pathway in adipocytes. The effect of Hcy on AMPK activation was subsequently determined. As shown in Fig. 4, A and B, exposure to Hcy for 2 h resulted in significantly elevated levels of phosphorylated AMPK under both basal and isoproterenol-stimulated conditions in 3T3-L1 adipocytes. The activation of AMPK by Hcy was confirmed via direct measurement of AMPK activity by ELISA (Fig. 4D) and corroborated by the significant increase in the phosphorylation levels of ACC and HSL at Ser565, the direct downstream targets of AMPK (Fig. 4, C and E). Furthermore, we examined the effects of Hcy (0.1 or 0.5 mM) on AMPK activation in primary adipocytes. As shown in Fig. 4F, Hcy treatment for 2 h elevated AMPK phosphorylation in primary adipocytes under basal conditions. A number of genes encoded for proteins involved in lipogenesis and lipolysis were also examined, and the results were shown in Fig. 4G. Among genes examined, only ACC expression was increased significantly by Hcy exposure.

Suppression of AMPK activation alleviates Hcy-mediated antilipolytic responses. To elucidate the role of the AMPK pathway in Hcy-suppressed lipolytic responses in adipocytes, we pretreated 3T3-L1 adipocytes with compound C (20 μM), a specific inhibitor of AMPK, and increasing concentrations of Hcy (0.05, 0.1, or 0.5 mM) for 2 h before isoproterenol stimulation for 15 min. Levels of glycerol and FFA release were determined. As shown in Fig. 5, A–D, addition of compound C reversed Hcy-induced decrease in glycerol and FFA release under both basal and isoproterenol-stimulated conditions, although isoproterenol stimulation markedly elevated intracellular cAMP levels compared with untreated cells. Similarly, Hcy failed to alter ERK1/2 phosphorylation and expression levels under both basal and isoproterenol-stimulated conditions (Fig. 3, B and C).
The mechanistic involvement of AMPK activation in Hcy-induced suppressive effect on lipolysis was further confirmed by AMPKα1 siRNA transfection experiments. As shown in Fig. 5, E and F, silencing AMPKα1 reversed Hcy-inhibited glycerol release in 3T3-L1 adipocytes.

**DISCUSSION**

Although long recognized as an independent risk factor for coronary artery disease, emerging evidence indicates that HHcy is also associated with adipose tissue dysfunction, including dysregulated adipokine production and decreased insulin sensitivity (10, 20, 28). The present study was conducted to examine the effect of Hcy on the lipolytic response in adipocytes and to explore the mechanistic pathways involved. Here, we provide initial evidence that Hcy exerts antilipolytic effects. We demonstrate that incubation with Hcy decreases glycerol and FFA release in fully differentiated 3T3-L1 and primary adipocytes in a dose-dependent manner under both basal and isopropoterenol-stimulated conditions. Inclusion of exogenous Hcy in the culture medium increases intracellular TG content in 3T3-L1 adipocytes, suggesting that reduction in FFA release is due to decreased lipolysis rather than increased oxidation of FFAs. Mechanistic investigations indicate that AMPK activation is critically involved in Hcy-induced inhibitory effects on lipolysis. Those in vitro observations were corroborated by our in vivo findings that dietary Hcy supplementation decreases fasting plasma glycerol and FFA levels in mice.

Several signaling pathways are involved in adipocyte lipolysis, with catecholamines being the most important lipolytic hormones. The major pathway leading to lipolysis is the cAMP-dependent PKA pathway (9), with evidence also implicating a critical role for the MEK-ERK1/2 pathway for maximal lipolytic activity on adrenergic stimulation (14). Because Hcy increases intracellular cAMP levels in hepatocytes (34), our initial hypothesis was that Hcy might promote lipolysis in...
Although initially controversial, more recent studies support that AMPK activation in adipocytes represents an important pathway to regulate lipolysis (1, 11, 12). Several lines of evidence demonstrated recently that activation of AMPK via either activators [metformin, 5-aminomimidazole-4-carboxamide-1β-n-ribonucleotide (AICAR), phenformin] (7, 12, 36) or the expression of a constitutively active form strongly inhibits isoproteolen-stimulated lipolytic processes. Conversely, lipolysis was enhanced in adipocytes expressing a dominant negative form of AMPK or isolated from AMPK-knockout mice (8, 35). Moreover, the activation of AMPK by isoproterenol, due to increased fatty acid reabsorption and metabolism, represents a feedback-inhibitory mechanism in regulating the lipolytic process (13). On the basis of these observations, we examined the effects of Hcy on AMPK activity in 3T3-L1 adipocytes. Our data revealed that Hcy treatment increased AMPK phosphorylation under both basal and isoproterenol-stimulated conditions. Time course study showed that isoproterenol-induced lipolysis was not observed until 30 min after its addition, which was concomitantly with AMPK activation (Supplemental Figure S1; Supplemental Material for this article is available online at the AJP-Endocrinology and Metabolism web site). This observation was consistent with the previous report (13). The increased AMPK activity was confirmed by direct activity measurement and corroborated by the concomitantly increased phosphorylation levels of ACC, a direct downstream target of AMPK. On the other hand, both compound C and AMPK gene silencing by siRNA transfection abolished the inhibitory activation of Hcy on glycerol and FFA release, indicating that AMPK activation plays a dominant role in Hcy-induced antilipolytic effects.

AMPK is a widely expressed serine/threonine kinase and considered to be an intracellular sensor of energy. AMPK is activated in response to an increase in the intracellular AMP/ATP ratio by an allosteric activation mechanism or by stimulating phosphorylation of the threonine residue 172 under conditions such as hypoxia, exercise, and long-term starvation (3, 15). Activation of AMPK leads to phosphorylation of a number of proteins, resulting in increased glucose uptake and fatty acid oxidation (16). With respect to its lipolytic action, AMPK activation was reported to increase phosphorylation of Ser565 on HSL, thereby reducing the translocation of endogenous HSL to the lipid droplet to initiate the lipolytic process (1). In the present study, we demonstrated increased HSL phosphorylation at Ser565 after Hcy exposure, indicating that AMPK activation plays a dominant role in Hcy-induced antilipolytic effects.

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Fig. 3. Hcy does not affect intracellular cAMP levels and ERK1/2 activation in 3T3-L1 cells. Differentiated 3T3-L1 adipocytes were pretreated with Hcy (0.1 or 0.5 mM) for 2 h and then exposed to 10 μM isoproterenol or vehicle for 15 min in fresh medium. A: adherent 3T3-L1 adipocytes were harvested for measurement of cAMP. B and C: ERK1/2 phosphorylation was assessed by Western blot (B) and quantified after normalization by total ERK1/2 expression (C). Data are means ± SD (n = 3). *P < 0.05 vs. UT.
remain elusive. Further investigations on the effects of Hcy exposure on intracellular AMP/ATP ratio or upstream kinase activities such as liver kinase B1 are required.

Hcy is a metabolite of the essential amino acid methionine and exists at a critical biochemical intersection in the methionine cycle. Since the liver is the major organ for methionine metabolism, disorders (both genetic and nutritional) leading to disrupted reactions in hepatic Hcy remethylation or its conversion to cysteine will cause excessive Hcy accumulation and secretion, resulting in so-called HHcy. Moreover, in a recent genome-wide association study, Souto et al. (29) identified nicotinamide N-methyltransferase (NNMT) as a potential genetic determinant of plasma Hcy levels. NNMT is an important cytosolic methyltransferase catalyzing the reaction of $S$-adenos-
sylmethionine with nicotinamide to yield \( S \)-adenosylhomocysteine, which is subsequently hydrolyzed to Hcy. In addition to its abundant expression in the liver, a recent study demonstrated that this enzyme is also expressed largely in mature adipocytes and contributed to increased adipose tissue Hcy production and secretion in response to nicotinamide overload (26). Thus, it is conceivable that, in addition to being a contributor to the plasma Hcy pool, under certain circumstances, such as NNMT substrate overload, local or intracellular Hcy levels in adipose tissue may become increased significantly above the micromolar range (20, 26), leading to regulation of the lipolytic process in a paracrine or autocrine manner in vivo.

In the present study, we also demonstrate that 2-wk Hcy feeding resulted in decreased fasting plasma glycerol and FFA levels in mice, indicative of a suppressed lipolytic process. Theoretically, a decreased lipolytic response may lead to increased adipose tissue mass, eventually leading to obesity. In our animal studies, although neither body weights nor epididymal fat mass/body weight ratios between two groups reached the statistical significance (data not shown), Hcy supplementation significantly elevated circulating leptin level and reduced adiponectin level. Moreover, the size of adipocytes from the Hcy group tends to be larger than in the control group. These data collectively suggest that elevated plasma Hcy levels may be associated with increased adiposity in this animal model. In fact, the association between HHcy and obesity has been reported, although conflicting results exist (6, 19, 21, 32). Long-term time course studies are required to establish a potential causal connection between increased Hcy and obesity. Moreover, the reported HHcy of obesity may worsen or accelerate the development of the metabolic syndrome via a
Fig. 6. Dietary Hcy supplementation lowers plasma glycerol and FFA levels and modulates adipokine production in mice. Male C57BL/6 mice were fed liquid diets with or without supplementation of 0.1% (wt/vol) Hcy for 2 wk. Levels of glycerol (A), FFAs (B), TG (C), cholesterol (D), leptin (E), and adiponectin (F) were measured in plasma. G: the gene expression of adiponectin and leptin in adipose tissue was detected via real-time RT-PCR. Levels of TG (H) and cholesterol (I) were also measured in liver homogenates. J: adipose tissue was stained with hematoxylin and eosin. *P < 0.05 vs. control. Data are means ± SD (n = 5).
vicious cycle. However, it is important to remember that a variety of factors contribute to the HHcy phenotype. Therefore, our in vivo observations may not extend to other experimental models. This heterogeneity may also account for controversial reports on the association between HHcy and obesity (6, 19, 21, 32).

In summary, here we report that Hcy exerts antilipolytic actions both in vivo and in vitro. Activation of AMPK by Hcy in adipocytes plays a critical role in the regulation of Hcy-induced inhibitory effects on lipolysis. Under physiological conditions, the lipolytic process is required to release energy resource in times of stress; therefore, inhibition of lipolysis is detrimental. However, in situations where obesity and insulin resistance, excess FFA release becomes harmful to other tissues or organs, suggesting that increased Hcy levels in adipose tissues might serve as a protective effect in obesity by inhibiting lipolysis.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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