

Enhancing hepatic glycolysis reduces obesity: Differential effects on lipogenesis depend on site of glycolytic modulation

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Summary

Reducing obesity requires an elevation of energy expenditure and/or a suppression of food intake. Here we show that enhancing hepatic glycolysis reduces body weight and adiposity in obese mice. Overexpression of glucokinase or 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase is used to increase hepatic glycolysis. Either of the two treatments produces similar increases in rates of fatty acid oxidation in extrahepatic tissues, i.e., skeletal muscle, leading to an elevation of energy expenditure. However, only 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase overexpression causes a suppression of food intake and a decrease in hypothalamic neuropeptide Y expression, contributing to a more pronounced reduction of body weight with this treatment. Furthermore, the two treatments cause differential lipid profiles due to opposite effects on hepatic lipogenesis, associated with distinct phosphorylation states of carbohydrate response element binding protein and AMP-activated protein kinase. The step at which hepatic glycolysis is enhanced dramatically influences overall whole-body energy balance and lipid profiles.

Introduction

Obesity is highly associated with type 2 diabetes and causes or exacerbates many health problems (Reaven, 1995). The mechanism for the pathogenesis of obesity remains unclear but in general terms is due to a loss of balance between energy intake (feeding) and energy expenditure (Spiegelman and Flier, 2001). Thus, obesity develops when energy intake chronically exceeds total body energy expenditure. Dysregulation of energy expenditure, at least in part, is attributable to impaired glucose and lipid metabolism. Notably, hyperinsulinemia, in combination with hyperglycemia, inhibits fatty acid oxidation in many tissues (Bavenholm et al., 2000; Chien et al., 2000; Rasmussen et al., 2002; Sidossis and Wolfe, 1996). As a result, lipogenesis is favored over fatty acid oxidation (Ruderman et al., 2003), leading to an increase in fat accumulation and a decrease in energy expenditure. Food intake, as the other component of energy balance, is controlled by the central nervous system (CNS), where the hypothalamus plays the most important role (Havel, 2001; Spiegelman and Flier, 2001). In hypothalamus, the arcuate nucleus (ARC) contains neurons expressing neuropeptide Y (NPY) and agouti-related protein (AgRP), which increase food intake, and neurons expressing pro-opiomelanocortin (POMC) and cocaine-amphetamine-related transcript (CART), which suppress food intake (Friedman, 2004). In genetic obese mice, hypothalamic NPY and/or AgRP mRNAs are elevated (Ollmann et al., 1997; Wilding et al., 1993). These changes are likely associated with increased food consumption (Williams et al., 2000).

One approach to reduce obesity is to elevate energy expenditure. In mice lacking acetyl-CoA carboxylase 2 (ACC2) (Abu-Elheiga et al., 2001), stearoyl-CoA desaturase-1 (SCD1) (Cohen

et al., 2002), or mice transgenic-expressing fibroblast growth factor-19 (FGF19) (Tomlinson et al., 2002), fatty acid oxidation is increased and whole-body energy expenditure is elevated. Consequently, fat mass and body weight are reduced or the development of obesity is prevented. In these studies, reduction of obesity involves a number of tissues, including liver, muscle, and brown adipose tissue, which cooperatively regulate whole-body fuel utilization. However, the relative role of individual tissues in the determination of whole-body energy expenditure is not well-defined. In addition, the above studies demonstrate that elevating fatty acid oxidation can improve glucose homeostasis. How enhancing glucose utilization affects lipid metabolism in terms of regulation of energy balance in obesity is not clear. Another approach for reduction of obesity is to suppress food intake. This is exemplified by peripheral administration of glucagon-like peptide-1 (GLP-1) derivative, NN2211, which reduces food intake and body weight in obese rats (Larsen et al., 2001). Since GLP-1 acts through binding to GLP-1 receptor in the CNS (Baggio et al., 2004), this study suggests that effects on the CNS are associated with a reduction of obesity through suppression of food intake.

Since insulin, in combination with glucose, is a major signal that regulates peripheral fatty acid oxidation (Bavenholm et al., 2000; Chien et al., 2000; Rasmussen et al., 2002; Sidossis and Wolfe, 1996), we hypothesized that lowering plasma insulin levels should be able to elevate energy expenditure and reduce obesity. Lowering plasma insulin levels can be achieved by reducing plasma glucose levels via suppression of hepatic glucose production (HGP). In the liver, glucokinase (GK) and 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (6PFK2/FBP2) are the two crucial enzymes that control glycolytic flux (Nordlie et al., 1999; Wu et al., 2005). GK catalyzes glucose phosphoryla-

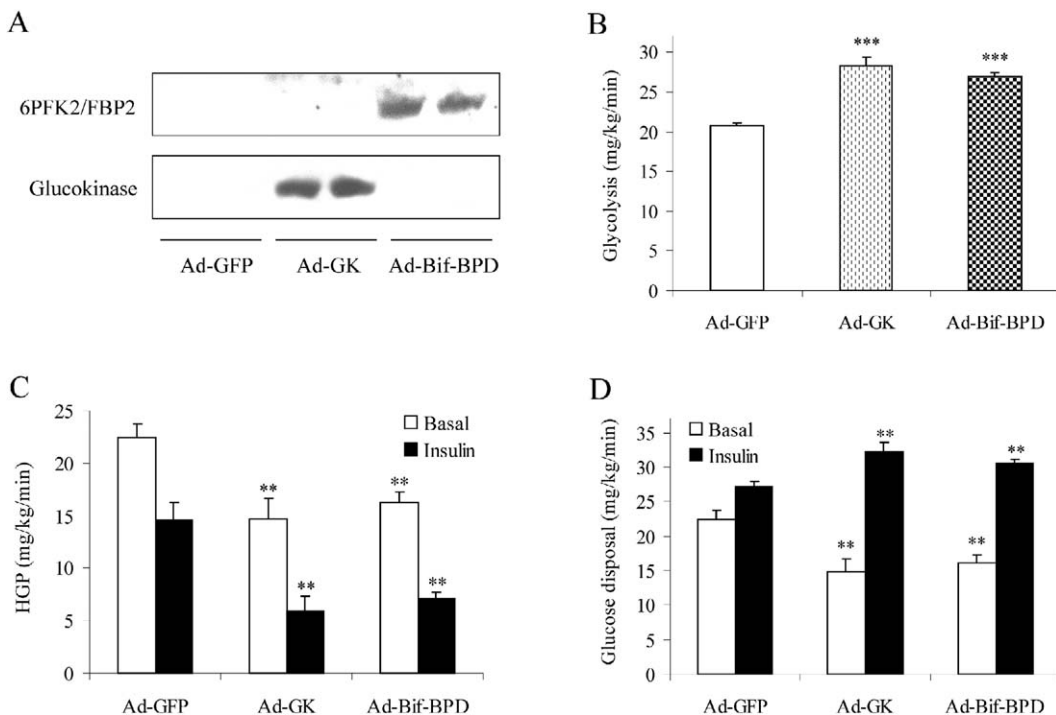


Figure 1. Enhancing hepatic glycolysis regulates whole-body glucose homeostasis

Obese KK/H1J mice were treated with adenovirus (Ad-GFP, Ad-GK, or Ad-Bif-BPD) on day 0 and followed for 7 days.

A) Hepatic overexpression of GK or 6PFK2/FBP2. In **(B)**, **(C)**, and **(D)**, data are means \pm SE, $n = 4-6$. ** $p < 0.01$ or *** $p < 0.001$ versus Ad-GFP.

B) Glycolysis.

C) Basal and insulin-suppressed hepatic glucose production (HGP).

D) Basal and insulin-stimulated whole-body glucose disposal (Rd).

tion as the first step of glucose metabolism, whereas 6PFK2/FBP2 (dephosphorylated hepatic isoform) elevates fructose-2,6-bisphosphate (F26P₂) to activate 6-phosphofructo-1-kinase (6PFK1) and promotes the step in glycolysis from fructose-6-phosphate (F6P) to fructose-1,6-bisphosphate (F16P₂). Thus, we chose to overexpress GK or 6PFK2/FBP2 in the liver of obese mice to explore the role of liver, particularly hepatic glycolysis, in the regulation of energy balance and obesity. In the course of these studies, we found dramatically different outcomes depending on the site of glycolysis targeted.

Results

Enhancement of hepatic glycolysis

In order to enhance hepatic glycolysis, Ad-GK or Ad-Bif-BPD were introduced into obese KK/H1J mice, a polygenic model of obesity and type 2 diabetes (Bouchard et al., 2002; Igel et al., 1998; Koishi et al., 2002; Nagase et al., 1996). Seven days after viral infusion, overexpression of either enzyme in livers of the treated mice was evident (Figure 1A). These manipulations caused a similar increase in hepatic glycolysis, which was evidenced by elevated levels of hepatic lactate (Table 1). In addition, whole-body glycolysis, measured during euglycemic/hyperinsulinemic clamp, was also increased similarly in Ad-GK- or Ad-Bif-BPD-treated mice (Figure 1B). Because adenoviral overexpression predominantly targets the liver (Becard et al., 2001; O'Doherty et al., 1999), enhanced glycolysis likely

occurred predominantly in the liver. This view was substantiated by the exclusive hepatic localization, in vivo, of green fluorescent protein (GFP) in the Ad-GFP-treated mice in the present study (data not shown). Upon enhancing hepatic glycolysis, both basal and insulin-suppressed HGP were decreased in Ad-GK- or Ad-Bif-BPD-treated mice compared to those of Ad-GFP-treated mice (Figure 1C). Whole-body glucose disposal, calculated as the rate of glucose disappearance (Rd), was decreased under the basal condition but increased under the hyperinsulinemic condition in Ad-GK- or Ad-Bif-BPD-treated mice (Figure 1D).

Reduction of body weight and adiposity

Upon enhancing hepatic glycolysis, Ad-GK- or Ad-Bif-BPD-treated KK/H1J mice showed reductions in body weight and adiposity, compared to Ad-GFP-treated mice (Figures 2A and 2B). Interestingly, Ad-Bif-BPD treatment produced much greater effects on reducing body weight than Ad-GK treatment. Since Ad-Bif-BPD treatment also suppressed food intake (see below), a group of Ad-GFP-treated mice were pair fed (Ad-GFP-PF) with mice treated with Ad-Bif-BPD. Food restriction caused a reduction of body weight and adiposity, but this effect was not as pronounced as that caused by Ad-Bif-BPD treatment (Figures 2A and 2B). Hence, the suppression of food intake only contributes partially to effects of Ad-Bif-BPD treatment on body weight loss.

Table 1. Changes in levels of hepatic metabolites and plasma parameters

	Ad-GFP	Ad-GK	Ad-Bif-BPD
Hepatic metabolites			
Lactate ($\mu\text{mole/g}$)	6.23 \pm 1.14	11.64 \pm 1.39*	9.49 \pm 0.83*
Triglycerides (mg/g)	20.12 \pm 1.60	79.19 \pm 3.04***	18.05 \pm 3.72***
G6P ($\mu\text{mole/g}$)	0.65 \pm 0.19	0.93 \pm 0.02***	0.70 \pm 0.01***
F6P ($\mu\text{mole/g}$)	0.19 \pm 0.01	0.26 \pm 0.02**	0.23 \pm 0.02
Xu5P (nmole/g)	57.13 \pm 7.45	87.29 \pm 10.43**	41.26 \pm 5.11**
Plasma parameters			
ALT (IU)	31.96 \pm 4.78	38.23 \pm 3.78	28.71 \pm 7.06
AST (IU)	37.43 \pm 4.33	31.06 \pm 5.62	32.56 \pm 4.09
Glucose (mg/dl)			
b	283.16 \pm 43.13	153.87 \pm 10.32*	148.47 \pm 9.41*
a	218.36 \pm 12.94	117.15 \pm 15.89**	115.45 \pm 9.05**
Triglycerides (mM)	2.11 \pm 0.10	6.45 \pm 1.71*	1.74 \pm 0.10* [‡]
Free fatty acids (mM)	2.04 \pm 0.25	3.30 \pm 0.67*	2.05 \pm 0.48 [‡]
Insulin (ng/ml)	9.70 \pm 1.24	4.57 \pm 0.23*	4.43 \pm 0.22*
Leptin (ng/ml)	13.30 \pm 0.83	0.33 \pm 0.40***	9.10 \pm 1.47* ^{‡‡}

Obese KK/H1J mice were treated with adenoviruses on day 0 and followed for 7 days. Hepatic metabolites were measured from frozen tissues that were collected on day 7 after 5 hr RQ experiment (5 hr fasting). Plasma glucose was monitored before (b) and after (a) RQ experiment. Other plasma parameters were measured only after RQ experiment. Data are means \pm SE, n = 5–8. *p < 0.05, **p < 0.01, or ***p < 0.001 versus Ad-GFP; and [‡]p < 0.05, ^{‡‡}p < 0.01, or ^{‡‡‡}p < 0.001 versus Ad-GK.

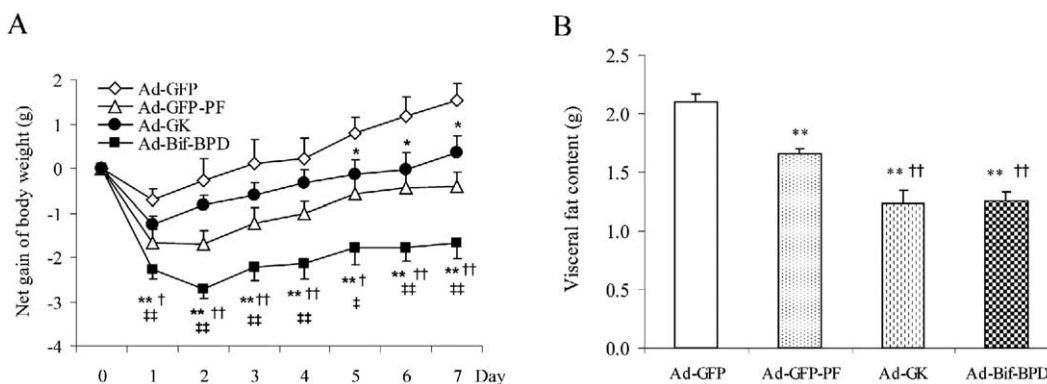
Elevation of whole-body energy expenditure

To test whether enhancing hepatic glycolysis leads to an increase in whole-body energy expenditure, the respiratory quotient (RQ) of virus-treated mice was measured on day 7. Ad-GK and Ad-Bif-BPD treatments caused identical reductions of RQ values compared to mice treated with control virus, Ad-GFP (Figure 3A). Food restriction did not change RQ values in the pair-fed mice. It should be noted that RQ values were lower in both Ad-GK- and Ad-Bif-BPD-treated mice than in Ad-GFP-treated mice at the initial time points. Moreover, RQ values were further reduced late in the 4 hr measurement when the mice were becoming fasted, compared to Ad-GFP-treated mice.

Energy expenditure, calculated from RQ data (Baar et al., 2004; Chen and Heiman, 2001), was elevated in both Ad-GK- and Ad-Bif-BPD-treated mice compared to Ad-GFP-treated mice (Figure 3B). The effects of Ad-GK treatment on energy

expenditure were greater than Ad-Bif-BPD treatment. In contrast, the pair-fed mice (Ad-GFP-PF) showed similar energy expenditure compared to Ad-GFP-treated mice, indicating that restriction of food intake does not affect whole-body fatty acid oxidation. There were no differences in the motion index of any treated mice, indicating that physical activities of all groups of treated mice were similar (data not shown).

We next measured palmitate oxidation rates of the liver and skeletal muscle to determine the contribution of individual tissues to the elevated whole-body fatty acid oxidation. Treatment with either Ad-GK or Ad-Bif-BPD caused a dramatic increase in the rates of palmitate oxidation in skeletal muscle (Figure 3C). In contrast, both treatments slightly decreased the rates of palmitate oxidation in liver. These data suggested that extra-hepatic tissues, including skeletal muscle, made a dominant contribution to an increase in whole-body fatty acid oxidation.

**Figure 2.** Enhancing hepatic glycolysis reduces body weight and adiposity

Obese KK/H1J mice were treated with adenovirus (Ad-GFP, Ad-GK, or Ad-Bif-BPD) on day 0 and followed for 7 days. A group of mice (Ad-GFP-PF) was treated with Ad-GFP and pair fed with Ad-Bif-BPD-treated mice. Data are means \pm SE, n = 5–8. *p < 0.05 or **p < 0.01 versus Ad-GFP, [‡]p < 0.05 or ^{‡‡}p < 0.01 versus Ad-GFP-PF, and [‡]p < 0.05 or ^{‡‡}p < 0.01 versus Ad-GK.

A) Body weight was monitored daily.

B) Epididymal, perinephric, and mesenteric fat depots were dissected and weighed as visceral fat content on day 7.

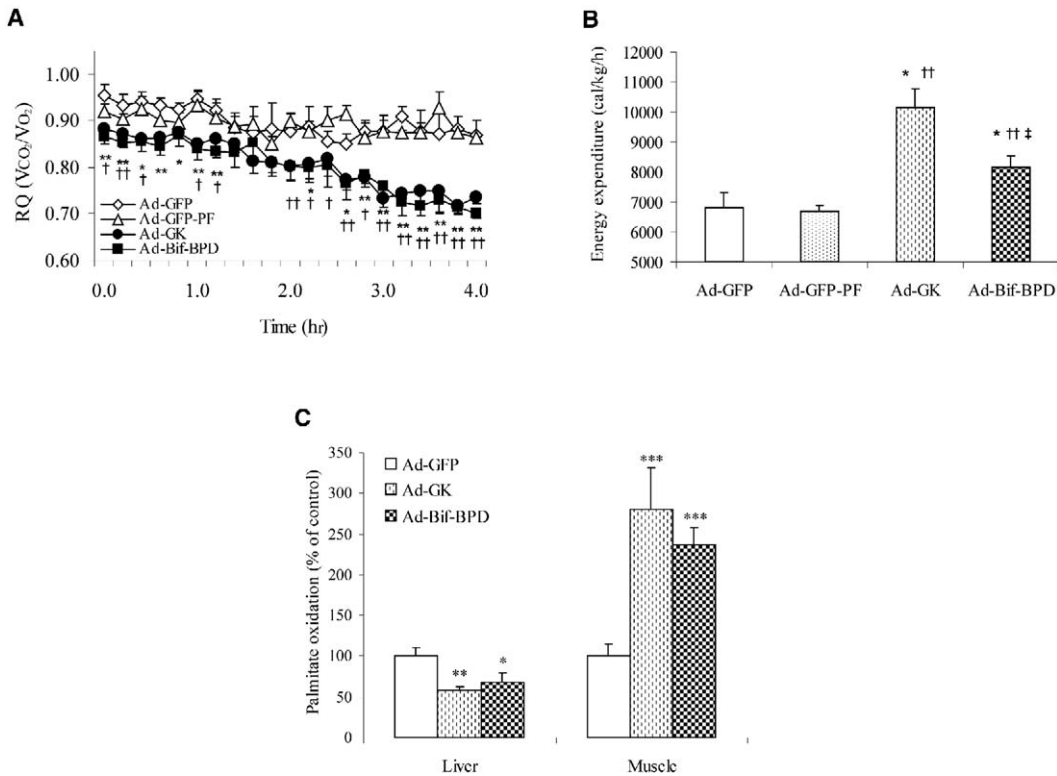


Figure 3. Enhancing hepatic glycolysis elevates energy expenditure

Obese KK/H1J mice were treated with adenovirus (Ad-GFP, Ad-GK, or Ad-Bif-BPD) on day 0 and followed for 7 days. A group of mice (Ad-GFP-PF) was treated with Ad-GFP and pair fed with Ad-Bif-BPD-treated mice. Data are means \pm SE, $n = 5-8$. * $p < 0.05$, ** $p < 0.01$, or *** $p < 0.001$ versus Ad-GFP; † $p < 0.05$ or †† $p < 0.01$ versus Ad-GFP-PF; and ‡ $p < 0.05$ versus Ad-GK.

A) RQ was measured on day 7 using indirect calorimetry.

B) Energy expenditure was calculated from RQ data.

C) Changes in the rates of palmitate oxidation. Results were expressed as the percentage of liver or muscle control.

Reduction of plasma levels of glucose and insulin

Enhancing hepatic glycolysis caused a suppression of HGP. This effect was thought to be responsible for lowering plasma glucose levels, which were observed with either Ad-GK or Ad-Bif-BPD treatment (Table 1). Due to these lower levels, glucose-stimulated insulin release (GSIR) is decreased in the pancreatic β cells, resulting in a subsequent reduction of plasma levels of insulin. Moreover, reductions of plasma levels of glucose and insulin were identical in the two liver-targeted manipulations. Initially, we used the decrease in plasma glucose and subsequent decrease in insulin levels as a simple explanation for the observed effects on promoting fatty acid oxidation in extrahepatic tissues. However, the dramatic differences in many aspects produced by the treatments strongly indicate that reducing obesity by enhancing hepatic glycolysis is a complicated process involving additional mechanisms.

Differential effects on food intake

Food intake is one of two determinants of energy balance. We monitored food intake of virus-treated mice daily. Ad-Bif-BPD treatment caused a suppression of food intake compared to Ad-GFP treatment, whereas Ad-GK treatment did not (Figure 4A). Thus, enhancing hepatic glycolysis at the step from glucose to G6P or from F6P to F16P₂ affects feeding behavior

differently. Because food intake is controlled by the CNS through integration of hormonal and nutritional signals (Flier, 2004; Havel, 2001), we next measured changes in mRNA levels of neuropeptides that control feeding behavior. Upon Ad-Bif-BPD treatment, the mRNA levels of hypothalamic NPY were decreased, suggesting that the CNS is secondarily affected. However, Ad-GK treatment did not affect NPY mRNA levels (Figure 4B).

Insulin may cooperate with leptin in the CNS signaling pathway for regulation of food intake (Flier, 2004; Havel, 2001; Spiegelman and Flier, 2001). Because plasma insulin levels were decreased similarly in both Ad-GK- and Ad-Bif-BPD-treated mice, we measured levels of plasma leptin. In the Ad-GFP-treated obese mice, plasma leptin levels were high. Upon Ad-Bif-BPD treatment, plasma leptin levels were moderately decreased (Table 1). Ad-GK treatment, surprisingly, produced a dramatic decrease in leptin levels. This decrease, however, was not accompanied by a change in food intake.

Differential effects on lipid metabolism

Although Ad-GK or Ad-Bif-BPD treatment produced identical effects on lowering plasma glucose and insulin levels, the two treatments caused strikingly different profiles of lipid metabolism. Upon Ad-GK treatment, the liver showed a 2- to 3-fold

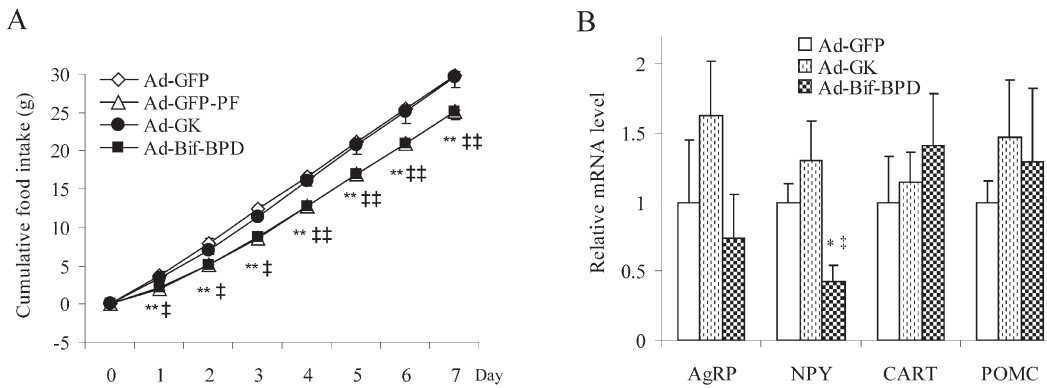


Figure 4. Enhancing hepatic glycolysis affects food intake and mRNAs of hypothalamic neuropeptides

Obese KK/H1J mice were treated with adenovirus (Ad-GFP, Ad-GK, or Ad-Bif-BPD) on day 0 and followed for 7 days. Data are means \pm SE. * $p < 0.05$ or ** $p < 0.01$ versus Ad-GFP and † $p < 0.05$ or †† $p < 0.01$ versus Ad-GK.

A) Cumulative food intake ($n = 5-8$). A group of mice (Ad-GFP-PF) was treated with Ad-GFP and pair fed with Ad-Bif-BPD-treated mice. The line indicating changes in food intake of Ad-GFP-PF-treated mice was superimposable on that of Ad-Bif-BPD-treated mice due to pair feeding.

B) Changes in mRNA levels of hypothalamic AgRP, NPY, CART, and POMC ($n = 3$). The mRNA levels were determined by real-time PCR.

increase in the size and a distinct pale-to-white color that was different from that of mice in all other groups (Figure 5A). These changes were consistent with 4-fold increases in hepatic triglycerides (TG, Table 1). Ad-Bif-BPD treatment, in contrast, did not change the weight or appearance of liver and did not influence hepatic TG levels. In addition, Ad-GK treatment exacerbated the existing hyperlipidemia in the KK/H1J mice, elevating plasma levels of triglycerides (TG) by 3-fold, whereas Ad-Bif-BPD treatment decreased plasma TG levels (Table 1). To test whether hepatic lipogenesis was responsible for changes in the lipid profile, we measured the *in vivo* rates of lipogenesis in liver. As expected, Ad-GK treatment caused a significant increase in hepatic lipogenesis. However, Ad-Bif-BPD treatment caused a decrease in hepatic lipogenesis (Figure 5B). Similar effects were also observed in rat primary hepatocytes (Figure 5B), indicating that the outcome of elevating glycolysis on lipogenesis is dramatically different depending on which step of glycolysis is targeted.

To understand the underlying mechanism, we measured changes in mRNA levels of lipogenic enzymes, acetyl CoA carboxylase 1 (ACC1), and fatty acid synthase (FAS). In the liver, ACC1 and FAS mRNA levels were increased in Ad-GK-treated mice, whereas they were decreased in Ad-Bif-BPD-treated mice (Figure 5C). Similar effects have been observed in Ad-GK- or Ad-Bif-BPD-treated normal 129J mice (J.E.K., C.W., C.J.H., B. Clem, and A.J.L., unpublished data). Unlike changes in the liver, ACC1 and FAS mRNA levels were decreased in skeletal muscle upon Ad-GK or Ad-Bif-BPD treatment (Figure 5D).

Transcription factor carbohydrate response element binding protein (ChREBP) is thought to mediate effects of glucose on induction of gene expression of lipogenic enzymes in the liver (Stoekman and Towle, 2002; Uyeda et al., 2002). Since hepatic ChREBP mRNA levels were decreased with either Ad-GK or Ad-Bif-BPD treatment (data not shown), we measured changes in ChREBP protein amount and phosphorylation state. In all virus-treated mice, ChREBP protein amounts were nearly the same. However, phosphorylation of ChREBP was

dramatically decreased in the liver of Ad-GK-treated mice and was not significantly changed in the liver of Ad-Bif-BPD-treated mice (Figure 5E). The decreased phosphorylation of ChREBP was likely associated with an elevation of hepatic levels of xylulose-5-phosphate (Xu5P), a regulatory metabolite (see below).

Since AMP-activated protein kinase (AMPK) plays an important role in regulation of lipid synthesis in the liver, we also measured changes in protein amount and phosphorylation state of AMPK. In the liver of Ad-GK-treated mice, protein amount and phosphorylation of AMPK were dramatically decreased (Figure 5E). These decreases were not observed with Ad-Bif-BPD treatment. Instead, AMPK phosphorylation was increased. Clearly, enhancing hepatic glycolysis at two distinct steps resulted in opposite effects on AMPK expression and phosphorylation.

Differential effects on hepatic G6P and Xu5P levels

Hepatic levels of G6P and Xu5P are closely associated with lipid metabolism in the liver (Kabashima et al., 2003; Towle et al., 1997). Thus, we measured changes in their levels (Table 1). With Ad-GK treatment, the hepatic levels of both G6P and Xu5P were increased ($0.93 \pm 0.03 \mu\text{mol/g}$ and $87.29 \pm 10.43 \text{ nmol/g}$, respectively) compared to those of mice treated with Ad-GFP ($0.65 \pm 0.19 \mu\text{mol/g}$ and $57.13 \pm 7.45 \text{ nmol/g}$, respectively). The increases in G6P were consistent with the effects of GK on phosphorylation of glucose, while the increases in Xu5P were likely associated with increased flux through pentose phosphate pathway due to the elevation of G6P (Kabashima et al., 2003). However, with Ad-Bif-BPD treatment, the hepatic levels of the two metabolites were not significantly changed.

Discussion

Increasing peripheral fatty acid oxidation can elevate energy expenditure and lead to a reduction or a prevention of obesity (Abu-Elheiga et al., 2001; Cohen et al., 2002; Tomlinson et al.,

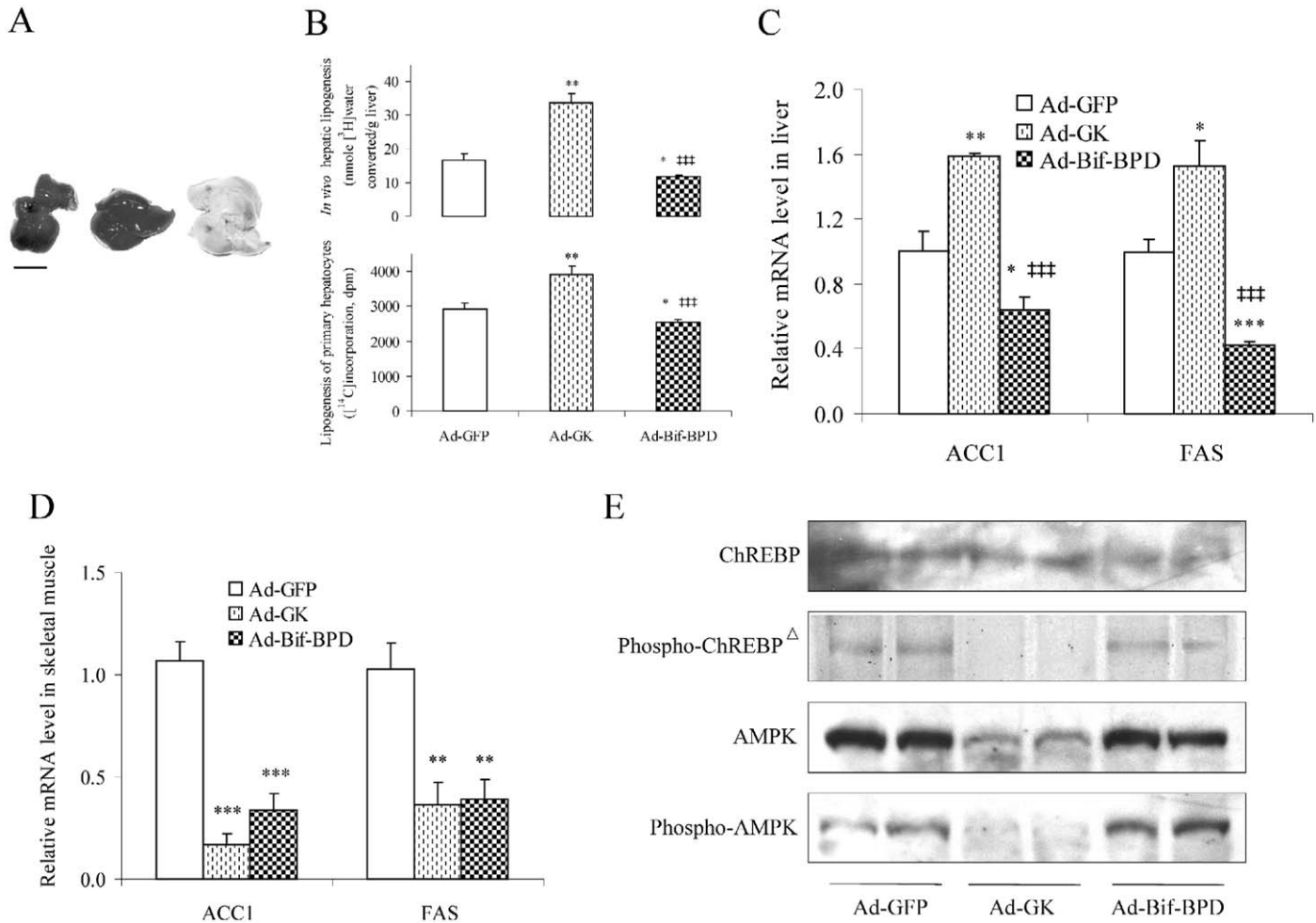


Figure 5. Enhancing hepatic glycolysis alters lipid metabolism

A) Changes of liver appearance. Livers from Ad-Bif-BPD-, Ad-GFP-, or Ad-GK-treated mice (from left to right) were collected on day 7. The dark bar is at a length of 1 cm. In (B), (C), and (D), data are means \pm SE. * $p < 0.05$, ** $p < 0.01$, or *** $p < 0.001$ versus Ad-GFP, and ## $p < 0.001$ versus Ad-GK.

B) Changes in lipogenesis. Top, in vivo lipogenesis in the liver ($n = 3-4$); bottom, lipogenesis in rat primary hepatocytes (measured from three to four independent experiments).

C) Changes in mRNA levels of ACC1 and FAS in the liver ($n = 5-8$).

D) Changes in mRNA levels of ACC1 and FAS in skeletal muscle ($n = 5-8$).

E) Changes in protein amount and phosphorylation of ChREBP and AMPK in the liver. Δ Phospho-ChREBP was analyzed from immunoprecipitation complex using phospho-protein gel stain.

2002). Because insulin, along with glucose, controls peripheral fatty acid oxidation and plasma levels of glucose, and because insulin can be manipulated secondarily via modulation of HGP, we used adenoviral overexpression of GK or a mutated form of 6PFK2/FBP2 to test whether and how enhancing hepatic glycolysis reduces obesity. Using these two manipulations, we were able to enhance glycolysis predominantly in the liver. Significantly, both of these manipulations resulted in identical glucose and insulin lowering effects. Because of this, we were able to dissect hepatic glycolysis at different steps and explore the role of each in the regulation of whole-body energy balance. These novel studies highlight both the central role of the liver in systemic energy balance and the mechanisms for differential regulation of hepatic lipogenesis.

Upon hepatic overexpression of either GK or 6PFK2/FBP2,

glycolysis was increased similarly in the liver, as indicated by elevated hepatic lactate levels. In addition, the rates of in vivo glycolysis were also increased similarly. These effects led to a similar decrease in HGP, which resulted in a similar reduction of levels of plasma glucose and, secondarily, a similar reduction of levels of plasma insulin. As a result, there was a relief of the inhibition of hyperinsulinemia on lipolysis in adipose tissue and fatty acid oxidation in muscle (Chien et al., 2000; Rasmussen et al., 2002; Sidossis and Wolfe, 1996; Stumvoll et al., 2000). In addition, when HGP was decreased, whole-body glucose disposal was also reduced, indicating a decrease in glucose utilization in peripheral tissues, i.e., muscle. From this perspective, an increase in fat utilization in muscle was expected to compensate for a reduction of glucose as a fuel. This change was reflected by decreases in the RQ values. As

expected, visceral fat content was decreased and the rates of fatty acid oxidation in skeletal muscle were increased in the treated mice, which contributed to the elevation of energy expenditure. Since the observed effects were nearly identical with GK or 6PFK2/FBP2 overexpression, the underlying mechanism(s) is likely the same. Therefore, reducing hyperinsulinemia secondary to lowering hyperglycemia by enhancing hepatic glycolysis may be the main contributor to an elevation of energy expenditure. This mechanism suggests that lowering plasma glucose levels likely mediates the liver-initiated intertissue interaction with extrahepatic tissues, such as pancreatic β cells, adipose, and skeletal muscle.

When energy expenditure was similarly elevated, a similar reduction of obesity was also expected. However, this was not the case. 6PFK2/FBP2 overexpression caused a more pronounced effect on reduction of body weight than did GK overexpression. This difference was attributable to differential effects of the two treatments on food intake. 6PFK2/FBP2 overexpression led to a suppression of food intake that was not observed with GK overexpression. When energy expenditure is elevated, food intake is usually increased due to counterregulation, which contributes to decreased effectiveness in reduction of obesity (Jequier, 2002; Perseghin, 2001). In this regard, enhancing hepatic glycolysis at the step from F6P to F16P₂ promoted cooperative regulation of energy balance to reduce obesity. Since food intake is controlled by the CNS (Flier, 2004; Havel, 2001), it is likely that 6PFK2/FBP2 overexpression led to secondary effects on the CNS. This view was supported by a decrease in hypothalamic NPY mRNA levels. With adenoviral overexpression, the initial alterations occurred in the liver (Becard et al., 2001; O'Doherty et al., 1999). Thus, liver-derived signals, neuronal, hormonal, or metabolic, might contribute to observed effects on food intake or hypothalamic NPY mRNA levels. In addition, the generation of liver-derived signals was likely determined by how hepatic glycolysis is enhanced. In the liver, AMPK, a key cellular energy sensor (Kahn et al., 2005), showed increased phosphorylation upon 6PFK2/FBP2 overexpression. Thus, it might serve as one of the signals to trigger an unknown hepatic neuronal sensor (or sensors) that is (are) connected with the CNS. Alternatively, 6PFK2/FBP2 overexpression might generate liver-derived signals, i.e., unknown protein(s), which would be secreted into the bloodstream and affect the CNS.

TG and leptin similarly may serve as circulating signals that impact the CNS. In fact, plasma TG levels correlated positively with NPY mRNA levels in all virus-treated mice. Leptin levels did not but the ratios of leptin to insulin did correlate negatively with NPY mRNA levels (data not shown). These correlations were consistent with the observed effects on food intake. In addition, leptin may cooperate with insulin on affecting the CNS. Since insulin stimulates leptin gene expression (Havel, 2001), a reduction of plasma insulin levels was expected to cause a decrease in plasma levels of leptin. This decrease was observed with either GK or 6PFK2/FBP2 overexpression. However, the two treatments also caused dramatic differences in leptin levels even though insulin levels were the same. These differences suggested that an additional factor (or factors) is involved in the regulation of leptin expression. Regardless of the mechanism (or mechanisms) for reduction of food intake, decreases in hypothalamic NPY mRNA levels serve as evidence of the existence of intertissue communication from the

liver to the brain, functioning as an additional component of the brain-liver circuit as proposed by Rossetti and colleagues (Pocai et al., 2005). It should be pointed out that we can not completely rule out the possibility that there may be a small amount of infection of the CNS by adenovirus treatment. Even if this is the case, it is likely that GK and 6PFK2/FBP2 overexpression generate different central signals for regulation of feeding.

Given the fact that GK or 6PFK2/FBP2 overexpression caused similar changes in rates of glycolysis but different consequences on the lipid profile and reduction of obesity, it is clear that the site at which glycolysis is enhanced in the liver can influence the outcome of whole-body lipid metabolism, as well as whole-body energy balance. This is particularly true with regard to the correlation between plasma TG levels and hypothalamic NPY mRNA levels. Since plasma TG levels were closely associated with hepatic lipogenesis, how lipogenesis was regulated in the liver was the key. Here, the opposite effects on ACC1 and FAS mRNAs contributed to differential regulation of hepatic lipogenesis. In light of the fact that gene expression of ACC1 and FAS is regulated by ChREBP and dephosphorylation of ChREBP is controlled by Xu5P-activated PP2A (Kabashima et al., 2003), a model for the underlying mechanism(s) was proposed. In this model, GK overexpression caused an increase in Xu5P levels through the pentose phosphate pathway. This increase activated PP2A, which led to an activation of ChREBP by removal of inhibitory phosphate(s). As a result, expression of ACC1 and FAS was increased. In addition, NADPH, another key product of the pentose phosphate pathway required for lipogenesis, was also likely increased to promote lipogenesis. None of these changes occurred upon 6PFK2/FBP2 overexpression. Instead, levels of G6P, Xu5P, and ChREBP phosphorylation were changed in a pattern favoring a decrease in lipogenesis, consistent with the proposed model.

AMPK also controls lipid synthesis in the liver. Activation of AMPK by phosphorylation inhibits ACC1 (Kahn et al., 2005). In addition, phosphorylation of AMPK inhibits the binding of ChREBP to DNA, which is required for induction of expression of genes for lipogenic enzymes (Kawaguchi et al., 2002). Upon enhancing glycolysis via GK overexpression, hepatic AMPK was dramatically decreased in both protein amount and phosphorylation. These changes are expected to cause a decrease in inhibition of ACC1 and an increase in the binding of ChREBP to DNA. As a result, lipid synthesis would be favored in the liver, which is what was observed. Upon enhancing hepatic glycolysis via 6PFK2/FBP2 overexpression, the increased phosphorylation of hepatic AMPK and decreased mRNAs of ACC1 and FAS were consistent with the AMPK-related mechanisms for a repression of lipid synthesis. How these opposite effects on hepatic AMPK were produced was not clear.

In conclusion, the liver plays an important role in the regulation of energy balance, and this is demonstrated by the reduction of obesity brought about by enhancing hepatic glycolysis. In addition, how hepatic glycolysis is enhanced determines the overall outcome of whole-body energy balance, evidenced by the fact that GK or 6PFK2/FBP2 overexpression caused differential effects on reduction of body weight and changes of lipid profiles in obese mice. When hepatic glycolysis is enhanced at the downstream step, energy balance is cooperatively regulated via the intertissue interactions between the liver and extrahepatic tissues. Through suppression of HGP, en-

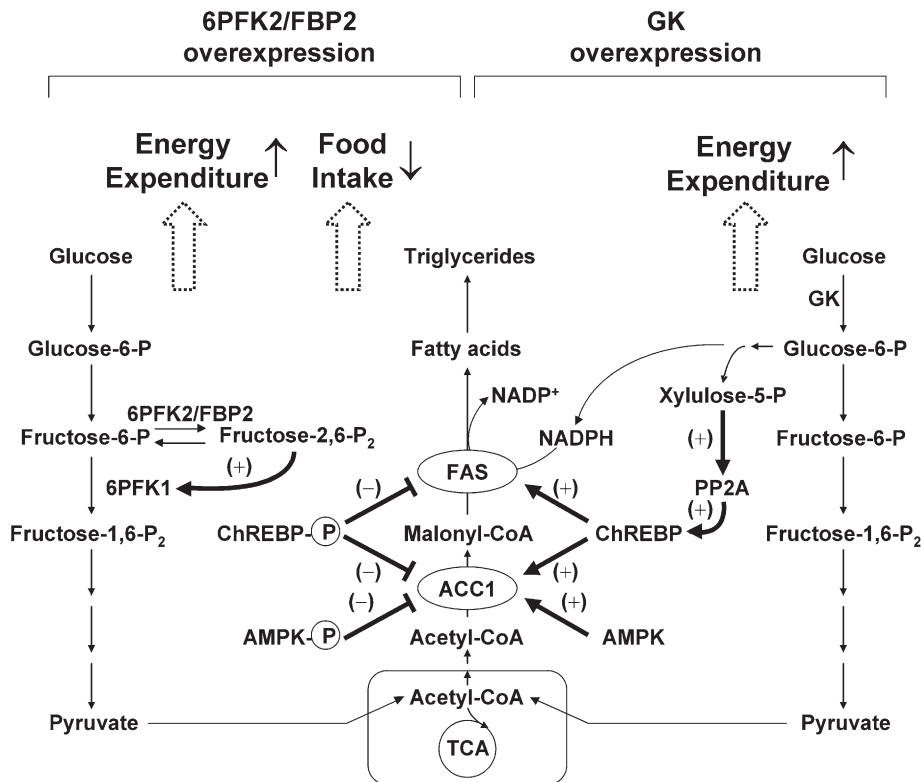


Figure 6. The mechanistic scheme for regulation of energy balance by enhancing hepatic glycolysis

Enhancing hepatic glycolysis via GK or 6PFK2/FBP2 overexpression causes an elevation of energy expenditure, likely through effects secondary to lowering plasma insulin and glucose levels. However, the two modulations cause opposite consequences on lipid profiles as well as differences in food intake. On the right side, when hepatic glycolysis is enhanced at the step from glucose to glucose-6-phosphate, hepatic lipogenesis is increased. On the left side, when hepatic glycolysis is enhanced at the step from fructose-6-phosphate to fructose-1,6-bisphosphate, food intake is suppressed and hepatic lipogenesis is decreased. Thus, when glycolysis is enhanced at the downstream step, the liver plays an important role in cooperative regulation of energy balance to reduce obesity.

hancing hepatic glycolysis at the step from F6P to F16P₂ leads to a decrease in plasma insulin levels secondary to a reduction of plasma glucose levels, which in turn promotes fatty acid oxidation in extrahepatic tissues, i.e., skeletal muscle, and elevates whole-body energy expenditure. In addition, unknown liver-derived signal (or signals) is likely generated uniquely in response to enhancing hepatic glycolysis at the downstream step and secondarily affects the hypothalamus, contributing to a suppression of food intake. The mechanistic scheme is summarized in Figure 6, showing how GK and 6PFK2/FBP2 overexpression regulates energy balance differently.

Experimental procedures

Adenovirus preparation

Adenoviruses encoding green fluorescent protein (Ad-GFP), glucokinase (Ad-GK), and bisphosphatase-deficient 6PFK2/FBP2 (Ad-Bif-BPD) were prepared as previously described (O'Doherty et al., 1999; Wu et al., 2001, 2004). The expressed bisphosphatase-deficient 6PFK2/FBP2, possessing a serine-32 to alanine mutation and a histidine-258 to alanine mutation, was designed to generate a higher level of F26P₂ compared to wild-type enzyme.

Animal treatments

Male obese KK/H1J mice were 12–14 weeks old (~35 g) and obtained from the Jackson Laboratory (Bar Harbor, Maine). All mice were fed ad libitum and individually housed and maintained on a 12:12 hr light-dark cycle, with lights turned on at 06:00. Adenovirus (Ad-GFP, Ad-GK, or Ad-Bif-BPD) was administered at 17:00 of day 0 via tail vein at a dose of $\sim 5 \times 10^{11}$ pfu/ml \times 0.45 ml. Body and food weight were measured daily at 10:00 on day -2 through day 7. The pair-fed mice (treated with Ad-GFP) were provided with the same amount of food as voluntarily ingested the previous day by Ad-Bif-BPD-treated mice at 17:00 on day -2 through day 7. At day 7, one set

of mice comprised of those treated with Ad-GFP, Ad-GK, Ad-Bif-BPD, and Ad-GPF-PF ($n = 5-8$) were used for RQ measurements. After RQ experiments, blood samples were collected from the tail vein, and mice were immediately sacrificed via cervical dislocation. The abdomen was quickly opened. Epididymal, perinephric, and mesenteric fat depots were dissected and weighed as visceral fat content (Barzilai et al., 1999). After weighing, the liver and skeletal muscle were rapidly trimmed and divided into two parts. One part of each tissue was frozen in liquid nitrogen and subsequently stored at -70°C for further analyses. The second part of the liver or skeletal muscle sample was immediately cooled in ice-cold buffer and homogenized. Tissue homogenates were used for determination of fatty acid oxidation as described below. The hypothalamic regions were dissected from 1 mm thick sagittal sections of fresh brain (Minokoshi et al., 2004). The arcuate nucleus (ARC), paraventricular (PVN), ventromedial (VMH), and dorsomedial (DMH) were collected microscopically, rapidly trimmed and frozen in liquid nitrogen, and subsequently stored at -70°C for further analyses. Two additional sets of mice treated with Ad-GFP, Ad-GK, and Ad-Bif-BPD ($n = 4-6$) were used separately for euglycemic/hyperinsulinemic studies and measurement of in vivo lipogenesis. All study protocols were reviewed and approved by the Institutional Animal Care and Use Committees of the University of Minnesota.

Primary hepatocyte preparation and treatment

Primary hepatocytes were isolated from SD rats as previously described (Stoeckman and Towle, 2002). Hepatocytes were plated into 12-well plates ($\sim 7 \times 10^5$ cells/well) for 4 hr with Williams E media. After overnight incubation, cells were treated with Ad-GFP, Ad-GK, or Ad-Bif-BPD (3.5×10^7 pfu/well) for 2 hr in Williams E media without FBS or insulin. Cells were then washed with PBS and incubated in Williams E media without FBS or insulin overnight.

RQ measurement

Respiratory gas measurements were taken by indirect calorimetry using a computer-controlled, open circuit system (Applied Electrochemistry Inc, Pittsburgh, Pennsylvania). Measurements were made at 10:00 on day 7.

After 1 hr to acclimate to the chamber, gases were sampled from the chamber every 2 min for the remaining 4 hr. During the entire 5 hr RQ measurement, food was restricted. The system then calculated the volumes of O₂ consumed (VO₂, ml/kg body weight) and CO₂ produced (VCO₂, ml/kg body weight) by the mouse every 2 min (Baar et al., 2004; Chen and Heiman, 2001). RQ is the ratio of VCO₂ to VO₂. Energy expenditure is calculated as EE = (3.185 + 1.232 × RQ) × VO₂ (Chen and Heiman, 2001).

Euglycemic/hyperinsulinemic clamp studies

An infusion catheter was inserted into a jugular vein of adenovirus-treated mice on day 5 (Rossetti et al., 1997). The clamp studies were conducted in conscious mice on day 7 after 5 hr fasting. The procedure included an 80 min basal and a 90 min hyperinsulinemic clamp period for assessment of the rates of glucose turnover, which were measured with prime-continuous infusion of [3-³H]glucose (Muse et al., 2004; Rossetti and Giaccari, 1990; Rossetti et al., 1997).

Measurement of lipogenesis

The in vivo hepatic lipogenesis of adenovirus-treated mice was measured on day 7. Briefly, each mouse was injected intraperitoneally with 0.3 ml of normal saline containing 0.5 mCi of [³H]water/100 g body weight. Samples of liver (0.5 g/mouse) were collected 1 hr after injection. Livers were solubilized and heated at 90°C for 5 hr in a mixture of 1.5 ml 4 M KOH and 1.5 ml of 95% ethanol. The sterols and fatty acids were isolated as previously described (Jiang et al., 2005). The lipogenesis of virus-treated rat primary hepatocytes was measured during the last 3 hr incubation by adding [¹⁴C]sodium acetate (0.5 μCi/well) to incubation media (Jiang et al., 2005).

Determination of fatty acid oxidation

Fatty acid oxidation was determined in fresh tissue using palmitate oxidation method as previously described (Xu et al., 2003; Yu et al., 1997). The rates of palmitate oxidation were calculated from the sum of ¹⁴CO₂ and ¹⁴C-labeled perchloric acid-soluble products.

Quantitation of mRNA by real-time PCR

The mRNA levels were measured using real-time PCR as previously described (Wu et al., 2004). Primer sequences are available upon request. Total RNA was isolated from frozen liver, skeletal muscle, or hypothalamus using STAT 60 reagent according to the manufacturer's protocol (TEL-TEST B Inc., Friendswood, Texas). Reverse transcription-PCR reactions were performed using the One-Step RT-PCR kit using 250 ng of total RNA (Roche Diagnostics Corporation, Indianapolis, Indiana). A control cDNA was used for interplate calibration, and the variability in the initial quantities of cDNA was normalized using ribosomal 18S RNA amplifications. Results were obtained from individual mice from three different groups, each tested in triplicate. Results were expressed as arbitrary units indicating relative expression normalized to 18S RNA.

Western blot, immunoprecipitation, and phospho-protein gel stain

The whole-liver lysates were used for analyses of overexpression of GK or 6PFK2/FBP2 and changes in protein amount of ChREBP, AMPK, and phospho-AMPK by Western blot as previously described (Wu et al., 2004). To analyze phospho-ChREBP, liver lysates were incubated with anti-ChREBP antibody and protein G plus agarose. The immunoprecipitation complex was resolved by SDS-PAGE, which was then stained with the Pro-Q phospho-protein gel stain reagent (Molecular Probes, Inc., Eugene, Oregon). Antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, California) and Cell Signaling Technology, Inc. (Beverly, Massachusetts).

Measurement of metabolites, transaminases, and hormones

Hepatic levels of G6P, F6P, Xu5P, and lactate were assayed from frozen liver using methods previously described (Kauffman et al., 1969; Wu et al., 2001, 2004). Hepatic TGs were extracted and concentration determined according to the method described by Ran et al. (2004). Plasma levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured using colorimetric methods (Reitman and Frankel, 1957). The assays for concentrations of plasma insulin, leptin, glucose, TGs, and free fatty acids were performed by following protocols provided by EIA kits (American Laboratory Products Company, Windham, New Hampshire) and metabolic assay kits (Sigma, St. Louis, Missouri).

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