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Overexpression of AMPKα1 Ameliorates Fatty Liver in Hyperlipidemic Diabetic Rats

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5'-AMP-activated protein kinase (AMPK) is a heterotrimeric complex consisting of a catalytic (α) and two regulatory (β and γ) subunits. Two isoforms are known for catalytic subunit (α1, α2) and are encoded by different genes. To assess the metabolic effects of AMPKα1, we examined the effects of overexpression of adenoviral-mediated AMPKα1 in hyperlipidemic type 2 diabetic rats. The Otsuka Long-Evans Tokushima Fatty (OLETF) rat is an established animal model of type 2 diabetes that exhibits chronic and slowly progressive hyperglycemia and hyperlipidemia. Thirty five-week-old overt type 2 diabetic rats (n=10) were administered intravenously with Ad.AMPKα1. AMPK activity was measured by phosphorylation of acetyl CoA carboxlyase (ACC). To investigate the changes of gene expression related glucose and lipid metabolism, quantitative real-time PCR was performed with liver tissues. Overexpression of AMPKα1 showed that blood glucose concentration was decreased but that glucose tolerance was not completely recovered on 7th day after treatment. Plasma triglyceride concentration was decreased slightly, and hepatic triglyceride content was markedly reduced by decreasing expression of hepatic lipogenic genes. Overexpression of AMPKα1 markedly improved hepatic steatosis and it may have effective role for improving hepatic lipid metabolism in hyperlipidemic state.

Key Words: AMP-activated protein kinase (AMPK), AMPKα1 catalytic subunit, Adenovirus, Type 2 diabetes, OLETF, Fatty liver

INTRODUCTION

5'-AMP-activated protein kinase (AMPK) is thought to act as a cellular 'fuel gauge' or 'metabolic switch' and has been suggested to play an important role in glucose utilization and fatty acid oxidation (Hardie et al., 2003; Hardie, 2004). Hepatic AMPK activation leads to suppression of gluconeogenesis and has favorable effects on lipid metabolism disorders (Hardie and Carling, 1997; Muoio et al., 1999). These effects on glucose and lipid metabolism have led to the identification of AMPK as a major pharmacological target for the treatment of metabolic disorders.

Studies in animal models of type 2 diabetes have shown that the pharmacological activation of AMPK with 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR) or metformin decreases blood glucose levels and improves lipid profile (Fryer et al., 2002; Thong and Graham, 2002; Rutter et al., 2003). However, these compounds are not ideal for investigating the beneficial effects of AMPK activation because they may affect AMPK-independent pathways (Kawano et al., 1992). This study investigated the effects of AMPK activation via overexpression of AMPK itself.

AMPK is a heterotrimeric complex consisting of a catalytic (α) and two regulatory (β and γ) subunits. Two isoforms are known for catalytic subunit (α1, α2) and they are encoded by different genes (Hardie et al., 1998). Skeletal muscle predominantly expresses the α2-isoform, whereas the liver expresses approximately equal amounts of α1 and α2 (Windr and Hardie, 1999). In the present study, we investigated specific effects of AMPKα1 in glucose and lipid metabolism with hyperlipidemic type 2 diabetic rats. AMPKα1 expression decreased blood glucose levels and plasma insulin concentration. However, glucose tolerance was not completely recovered by overexpression of AMPKα1. Surprisingly, hepatic steatosis was markedly improved on 7th day after Ad.AMPKα1 treatment. To our knowledge, this is the first study to use Ad.AMPKα1 to modulate AMPK activity in order to determine the effects of specific catalytic subunit of the kinase on hyperlipidemic type 2 diabetes. The present study may provide valuable information for further elucidation of the role of AMPKα1 in lipid metabolic disorders.

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ABBREVIATIONS: AMPK, 5'-AMP-activated protein kinase; ACC, acetyl CoA carboxlyase; OLETF, Otsuka Long-Evans Tokushima Fatty; LETO, Long-Evans Tokushima Otsuka; HOMA, homeostatic medel assessment; FFA, free fatty acid.

METHODS

Animals

Male Otsuka Long-Evans Tokushima fatty (OLETF) rats and their lean non-diabetic counterparts, Long-Evans Tokushima Otsuka (LETO) rats, were supplied by Otsuka Pharmaceutical (Tokushima, Japan). Hyperlipidemic type 2 diabetic OLETF rats (n=10) aged 35 weeks old were intravenously administered the adenoviral-mediated AMPKα1 gene (Ad.AMPKα1, 2×10^{12} particles/rat). Adenoviral-mediated lacZ gene (Ad.lacZ) was used for the diabetic control group (n=10). All OLETF rats were cared for according to the Guidelines of Animal Experiments recommended by Korean Academy of Medical Sciences throughout the entire experimental period.

Recombinant adenovirus

Full-length myc epitope-tagged rat AMPKα1 cDNA was kindly provided by Dr. J. Ha (Kyung Hee University, Seoul, Korea) and then subcloned into the shuttle vector. Recombinant adenoviral vector was obtained as described for the BD Adeno-X^{TM} expression System 1 (BD Biosciences, Bedford, MA). Ad.AMPKα1 were propagated in 293 cells and purified by cesium chloride density centrifugation.

Non-radioactive AMPK activity assay

Liver extracts from Ad.AMPKα1-injected and control rats were added to assay buffer (20 mM MOPS, pH 7.2, 25 mM β-glycerolphosphate, 5 mM EGTA, 1 mM dithiothreitol). ACC2 (amino acid 175-271), the AMPK substrate, was then added to the buffer with or without AMPK (1mU/l) and Magnesium/ATP Cocktail (75 mM $MgCl_2$, 0.5 mM ATP in assay buffer). The samples were then incubated for 30~60 minutes at 30°C. Western blotting was performed with anti-phospho-ACC antibody (Upstate Biotechnology, Lake Placid, NY).

IVGTT and measurement of glucose, insulin, and lipid levels

After an 18-h fasting period, all animals were anesthetized with ether, and tail vein blood samples were collected to measure glucose, insulin, FFA, total cholesterol and triglyceride levels. For intravenous glucose tolerance tests (IVGTT), rats were injected with glucose (200 mg/kg) via tail vein, and blood samples were collected 0, 10, 30, 60, 90 and 120 minutes after the glucose load. The sampling line was filled with 4.5% EDTA to prevent blood clotting. Samples were kept on ice, and plasma was isolated and stored at −20°C until analysis. Glucose levels were measured with a glucose analyzer (Allmedicus, An-Yang, Korea). Insulin levels were analyzed with the rat insulin ELISA kit (Linco Research, St. Charles, MO). Total cholesterol and triglyceride levels were measured with the ASAN Set Total-cholesterol Reagent and TG-S Reagent (ASAN PHARM., Seoul, Korea), respectively. Free fatty acid levels were measured by the ACS-ACOD enzyme method (NEFA ZYME-S, Sinyang-Chemistry, Seoul, Korea).

Quantitative real-time PCR

Total RNA was isolated using TRIzol (Invitrogen, Carlsbad, CA). Complementary DNA was synthesized from RNA samples by mixing total RNA and oligo (dT) primers in the presence of 50 mM Tris-HCl buffer (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, 20 U enhanced MLV reverse transcriptase, 20 U of RNase inhibitor, and 0.5 mM of each dNTP. Real-time RT-PCR was carried out with SYBR Green Master mix (Applied Biosystems, Foster City, CA) and 10 pmol/l of primers in the 7300 Real-Time PCR System (Applied Biosystems). 18S RNA was used as the invariant control for all studies.

Histological analysis

To detect neutral lipids, liver cryosections were stained with Sudan Black B according to the manufacturer's protocol (Biogenex, San Ramon, CA). In brief, cryosections were incubated with Sudan Black for 3 min, rinsed twice and then incubated with 70% alcohol for 2 min and rinsed twice. Cryosections were counterstained with Nuclear Fast Red.

Analysis of hepatic lipids

Hepatocytes were lysed in ethanolic KOH (2 parts EtOH: 1 part 30% KOH) and incubated overnight at 55°C. The lysates were mixed with H_2O : EtOH (1 : 1) and centrifuged for 5 min. The supernatant was mixed with 1M $MgCl_2$, incubated for 10 min on ice and centrifuged for 5 min. The resulting supernatant was used to measure cholesterol and triglyceride.

Statistical analysis

Results are expressed as means±SEM. Differences between groups were analyzed by paired or non-paired Student's t test. Differences were considered significant when the p value was less than 0.05.

RESULTS

Expression of AMPKα1 in rats by adenoviral-mediated gene transfer

Tissue was analyzed on 7th day after Ad.AMPKα1 infection to assess its delivery and activity in rats. We checked for AMPKα protein activity in the liver of infected rats with a non-radioactive AMPK activity assay (Fig. 1A). AMPK activity levels were considerably increased by days 7 after Ad.AMPKα1 infection. We also assessed phosphorylation of AMPKα and ACC, a substrate of AMPK by Western blotting (Fig. 1B). Phosphorylation of ACC and AMPKα was noticeable by day 7 after Ad.AMPKα1 injection. We determined that the 7th day after Ad.AMPKα1 infection is the most effective for physiological action of AMPKα1. The present study examined the effects of Ad.AMPKα1 with liver tissue because adenoviral-mediated gene transfer is almost delivered to liver. Body weight and food intake were not affected by Ad.AMPKα1 infection until day 7 after adenoviral injection (data not shown).

Hepatic AMPKα1 expression does not fully improved glucose homeostasis in diabetic rats

We investigated the impact of hepatic AMPKα1 on glucose metabolism in vivo by injecting Ad.lacZ or Ad.AMPKα1 into

type 2 diabetic OLETF rats. As shown in Table 1, blood glucose concentration and plasma insulin levels were significantly decreased on 7th day after injection in Ad.AMPKα1-infected rats. Decreased plasma insulin concentration was associated with the physiological response of decreased blood glucose levels. HOMA index representing insulin resistance was also significantly decreased in Ad.AMPKα1-treated rats. However, HOMA index of Ad.AMPKα1-infected rats still represents insulin resistance. To determine the physiological consequences of Ad.AMPKα1 expression in diabetic OLETF rats, glucose tolerance tests were performed. Glucose tolerance seemed to be improved in Ad.AMPKα1-treated OLETF rats, similar to LETO rats (Fig. 2A). However, blood glucose levels of Ad.AMPKα1-treated OLETF rats were not recovered to initial levels until 120 minutes after glucose loading. Early response of insulin secretion (10 min) of Ad.AMPKα1-treated rats was analogous to Ad.lacZ-treated rats: plasma concentrations of insulin of Ad.AMPKα1-treated rats were paradoxically decreased even though the levels of blood glucose were increased after glucose loading (Fig. 2B). These results indicated that Ad.AMPKα1 decreased the blood glucose levels but that it could not regulate secretion of insulin.

The expression of genes encoding enzymes involved in gluconeogenesis and glycolysis was examined by quantitative real-time PCR in Ad.AMPKα1-infected rats (Fig. 2C). Expression of enzymes involved in gluconeogenesis, PEPCK, G6Pase and FBPase, showed significant difference only in G6Pase of Ad.AMPKα1-treated OLETF rats. Expression of other enzymes involved in glycolysis was not changed in Ad.AMPKα1-treated OLETF rats. Thus, overexpression of AMPKα1 was not sufficient to improve glucose homeostasis in type 2 diabetic rats.

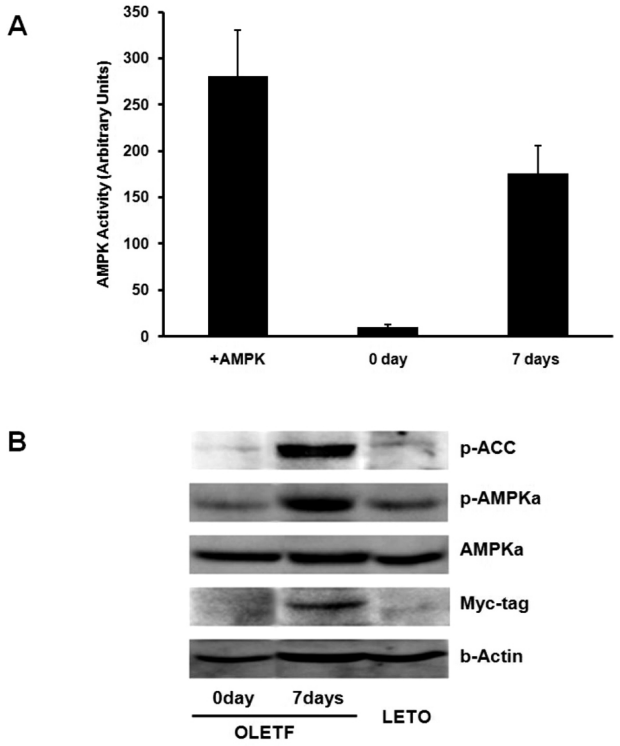

Fig. 1. Activity and expression of AMPK by Ad.AMPKα1 injection. (A) Non-radioactive AMPK activity assay. AMPK activity in the liver was measured on day 0 (0 day) and 7 (7 days) after Ad.AMPKα1 injection, and for the positive control with added AMPK recombinant protein (+AMPK). (B) Western blot analysis of phospho-ACC, phospho-AMPKα, AMPKα and myc-tagged AMPKα1 levels in liver on day 0 (0 day) and 7 (7 days) after Ad.AMPKα1 injection into OLETF rats. LETO, lean nondiabetic counterpart rats.

Hepatic AMPKα1 expression improves hepatic steatosis in hyperlipidemic diabetic rats

OLETF rats aged 35 weeks old showed hypertriglyceridemia and hypercholesterolemia (Table 1). Histological analysis with Sudan Black B staining showed that lipid droplets accumulated in hepatocytes in the pericentral regions of Ad.lacZ-injected OLETF rats (Fig. 3A, Ad.lacZ). LETO rats aged 35 weeks old also had fine fat globules. Ad.AMPKα1-injected OLETF rats showed significantly decrease of the amount of lipid droplets (Fig. 3A, Ad.AMPKα1). In agreement with these results, hepatic triglyceride levels of Ad.AMPKα1-injected OLETF rats decreased significantly, even below the levels in LETO rats (Table 1). In addition, plasma concentrations of triglyceride were significantly decreased in Ad.AMPKα1-injected rats. However, plasma levels of free fatty acid were not significantly changed and those of cholesterol were increased compared to Ad.lacZ-injected OLETF rats. To elucidate the mechanism by which Ad.AMPKα1 reduces hepatic lipid content in OLETF fatty liver, we examined the mRNA levels of hepatic acetyl-CoA carboxylase 1 (ACC-1, a key regulator of fatty acid synthesis/oxidation), fatty acid synthase (FAS, a key enzyme

Table 1. Metabolic characteristics of Ad.AMPKα1-injected OLETF rats

Parameter	OLETF		LETO
	Ad.LacZ	Ad.AMPKα1	
Glucose (mg/dl)	251±21	117±11**	123±9*
Insulin (ng/ml)	6.08±1.42	1.37±0.82**	0.84±0.34**
HOMA index	81.5±22.3	8.8±7.7**	5.4±2.0**
Plasma triglyceride (mg/dl)	367±41	272±74*	68±14**
Plasma FFA (uEq/l)	418±115	512±88	354±52
Plasma cholesterol (mg/dl)	150±34	188±13*	55±3*
Liver triglyceride (mg/g liver wt)	95.6±13.7	62.5±10.2*	72.2±23.2
Liver cholesterol (mg/g liver wt)	10.6±1.1	9.0±1.2	8.3±1.6

Data are means±SE. *$p<0.05$, **$p<0.01$ vs. control rats.

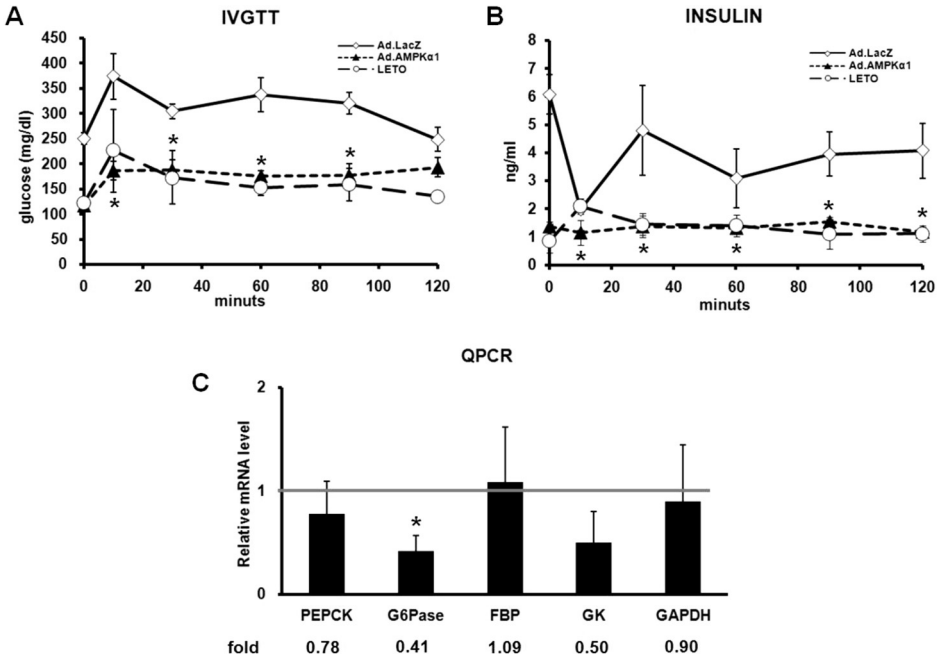

Fig. 2. Ad.AMPKα1 alters glucose metabolism. (A) Effect of Ad.AMPKα1 on glucose tolerance. Glucose tolerance was measured on days 7 after injection of Ad.lacZ (Ad.lacZ) or Ad.AMPKα1 (Ad.AMPKα1) into OLETF rats. LETO rats were employed as nondiabetic counterpart. (B) Response of insulin secretion to glucose loading. Plasma concentrations of insulin were measured on the same time of assessing blood glucose. (C) Quantitative RT-PCR analysis of enzymes related to gluconeogenesis and glycolysis. *Significantly different from Ad.lacZ-injected OLETF rats ($p<0.05$).

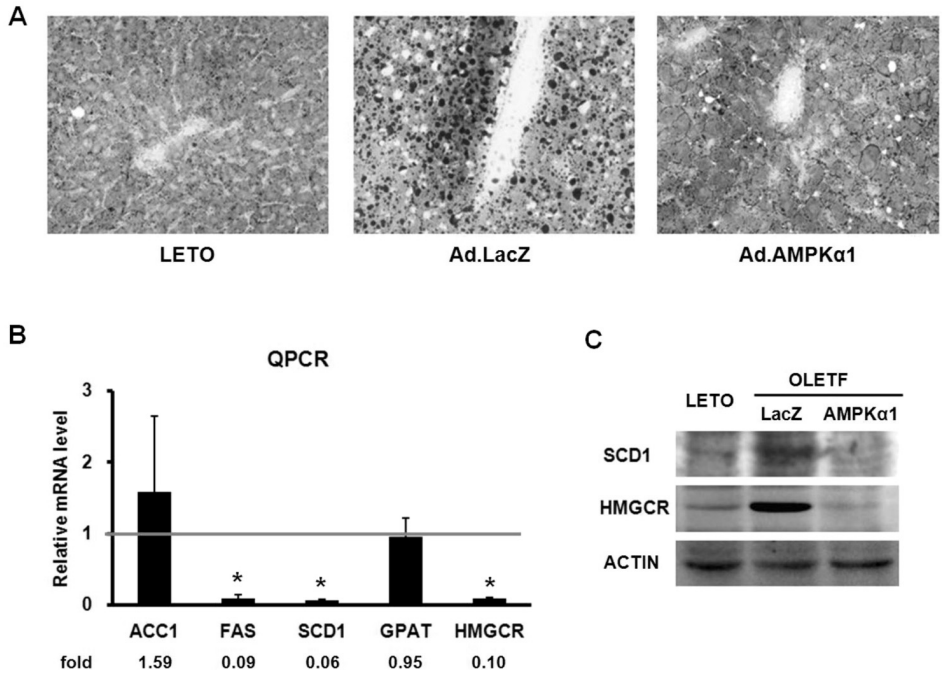

Fig. 3. Ad.AMPKα1 decreases lipid content in liver. (A) Sudan Black B stained liver cryostat section from normal LETO rats, Ad.lacZ-injected OLETF rats (Ad.lacZ) and Ad.AMPKα1-injected OLETF rats (Ad.AMPKα1). Magnification ×200. The liver of Ad.lacZ-injected OLETF rat shows accumulation of large fat globules (Black). The liver of Ad.AMPKα1-injected OLETF rat shows marked decrease of fat globules. (B) Quantitative RT-PCR analysis of enzymes related to lipid synthesis. (C) Western blot analysis of SCD1 and HMGCR in liver on day 7 after injection. *Significantly different from Ad.lacZ-injected OLETF rats ($p<0.05$).

in synthesis of fatty acid), stearoyl-CoA desaturase 1 (SCD1, a rate-limiting enzyme in the biosynthesis of monounsaturated fatty acids as major components of triglycerides), glycerol 3-phosphate acyltransferase (GPAT, an essential enzyme in triglyceride synthesis) and HMG-CoA reductase (HMGCR, a key enzyme in cholesterol synthesis). Activation of AMPK is known to phosphorylate and inhibit ACC1, leading to a decrease in malonyl CoA and an increase in fatty acid oxidation. Phosphorylation of ACC was increased in 7 day-Ad.AMPKα1-treated OLETF rats (Fig. 1B). However, the gene expression of ACC in Ad.AMPKα1-treated rats was not changed (Fig. 3B). Expressions of FAS, SCD1 and HMGCR were strongly inhibited in Ad.AMPKα1-treated OLETF rats (Fig. 3B), but that of GPAT showed no significance. Protein levels of SCD1 and HMGCR is decreased in Ad.AMPKα1-treated OLETF rats (Fig. 3C). Modulation of these enzymes by Ad.AMPKα1 may contribute to improvement of hepatic steastosis in hyperlipidmic type 2 diabetic rats.

DISCUSSION

Recent findings, which showed that hepatic AMPK is activated by adiponectin (Yamauchi et al., 2002), metformin (Zhou et al., 2001) and thiazolidinediones (TZDs) (Saha et al., 2004), reinforced therapeutic interest in metabolic disorders. Among chemical methods, AICAR has been used both in vitro (Corton et al., 1995) and in vivo (Pencek et al., 2005; Reiter et al., 2005) to activate AMPK. While AICAR is still the most widely used pharmacological activator of AMPK, some of its actions could be independent of AMPK (Corton et al.,1995; Rencek et al., 2005).

One important issue is the extent to which α1 and α2 catalytic subunits have specific targets. The α2 isoform has been proposed to play a major role in controlling metabolism, at least in skeletal muscle, but activation of the α1 isoform is also observed during some interventions in skeletal muscle (Musi et al., 2001; Minokoshi et al., 2002; Wojtaszewski et al., 2002). However, extensive analysis of specific catalytic isoform action in other tissue has not been performed. Indeed, most AMPK actions have been described after pharmacological activation of AMPK by AICAR. Because tissue effects observed upon AICAR administration may be the result of activation of both α1 and α2 catalytic isoforms, it is impossible to distinguish the specific actions of either isoform. To gain more insight into this issue, we therefore investigated the effects of AMPKα1 overexpression.

A recent study reported that expression of AMPKα2 leads to mild hypoglycemia and induces fatty liver in normal mice (Foretz et al., 2005). The present study investigated that expression of AMPKα1 caused decrease of blood glucose level and hepatic gluconeogenic gene expression, as did AMPKα2 (Foretz et al., 2005). However, hepatic lipid alteration revealed a clear distinction between α1 and α2 catalytic subunits, even though the animal models between two groups were different. While expression of AMPK α2 led to lipid accumulation in liver (Foretz et al., 2005), expression of AMPK α1 reduced the lipid content in fatty liver. Histological analysis with Sudan Black B staining revealed that large fat globules in hyperlipidemic liver almost disappeared by Ad.AMPKα1. Hepatic and circulating triglyceride levels were decreased in agreement with histological findings. However, the concentrations of cholesterol and free fatty acid were not affected by AMPKα1 expression, even though the expression of HMGCR was significantly reduced. Interestingly, infection with Ad.AMPKα1 led to the repression of SCD1 expression. SCD1 plays a crucial role in lipid metabolism and body weight control (Cohen et al., 2003; Dobrzyn and Ntambi, 2004). SCD1 deficiency reduces hepatic steatosis in lipodystrophic aP2-nSREBP-1c mice (Asilmaz et al., 2004). ACC1, a major enzyme in the regulation of cellular fatty acid synthesis (Hardie and Pan, 2002) was the first described in vivo target for AMPK, with its phosphorylation resulting in a decline in cytosolic malonyl-CoA concentration, which switches off fatty acid synthesis and augments mitochondrial uptake of fatty acids for β-oxidation (Winder et al., 1997). The present study demonstrated that Ad.AMPKα1 increased phosphorylation of ACC1 without affecting its mRNA levels. A recent report observed that hepatocytes from AMPKα1-knockouts have an almost total loss of ACC1 phosphorylation compared with AMPKα2 knockouts and wild-type (Crawford et al., 2006). The data indicate that AMPKα1 is the primary driver of ACC1 phosphorylation in liver cytosol. We suggest that improvement of hepatic steatosis results from repression of SCD1 expression and augmented phosphorylation of ACC1 by Ad.AMPKα1.

In conclusion, the metabolic consequences of hepatic expression of the AMPKα1 catalytic subunit alleviate hyperglycemia and improve fatty liver in hyperlipidemic diabetic rats. While the effects of Ad.AMPKα1 expression are similar to those of AMPKα2 expression in glucose metabolism, hepatic lipid alterations reveal the distinction between α1 and α2 catalytic subunits. Further investigation is needed to determine the molecular targets of α1 and α2 catalytic subunits in lipid metabolism, which will further elucidate the role of AMPKα1 and α2 in hyperlipidemic diabetes.

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