Original Article

Polyphenols Stimulate AMP-Activated Protein Kinase, Lower Lipids, and Inhibit Accelerated Atherosclerosis in Diabetic LDL Receptor–Deficient Mice

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Because polyphenols may have beneficial effects on dyslipidemia, which accelerates atherosclerosis in diabetes, we examined the effect of polyphenols on hepatocellular AMP-activated protein kinase (AMPK) activity and lipid levels, as well as hyperlipidemia and atherogenesis in type 1 diabetic LDL receptor–deficient mice (DMLDLR$^{-/-}$). In HepG2 hepatocytes, polyphenols, including resveratrol (a major polyphenol in red wine), apigenin, and S17834 (a synthetic polyphenol), increased phosphorylation of AMPK and its downstream target, acetyl-CoA carboxylase (ACC), and they increased activity of AMPK with 200 times the potency of metformin. The polyphenols also prevented the lipid accumulation that occurred in HepG2 cells exposed to high glucose, and their ability to do so was mimicked and abrogated, respectively, by overexpression of constitutively active and dominant-negative AMPK mutants. Furthermore, treatment of DMLDLR$^{-/-}$ mice with S17834 prevented the decrease in AMPK and ACC phosphorylation and the lipid accumulation in the liver, and it also inhibited hyperlipidemia and the acceleration of aortic lesion development. These studies *1*) reveal that inactivation of hepatic AMPK is a key event in the pathogenesis of hyperlipidemia in diabetes, *2*) point to a novel mechanism of action of polyphenols to lower lipids by activating AMPK, and *3*) emphasize a new therapeutic avenue to benefit hyperlipidemia and atherosclerosis specifically in diabetes via activating AMPK. *Diabetes* 55:2180–2191, 2006

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ACC, acetyl-CoA carboxylase; AICAR, 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside; AMPK, AMP-activated protein kinase; apo, apolipoprotein; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; GFP, green fluorescent protein; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; PPAR, peroxisome proliferator–activated receptor; Sirt1, sirtuin 1; STZ, streptozotocin; TBST, Tris-buffered saline with Tween.

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In diabetic patients atherosclerosis and its clinical complications are dramatically accelerated. This has been attributed to the effects on the vascular wall of the diabetic milieu, which include hyperglycemia, dyslipidemia, inflammation, and oxidant stress (1). In both type 1 and type 2 diabetes, treatment with hydroxymethylglutaryl-CoA reductase inhibitors and peroxisome proliferator–activated receptor (PPAR)γ agonists lowers lipids, which contributes to ameliorating the acceleration of atherosclerosis (2). Metformin, an activator of AMP-activated protein kinase (AMPK) (3–5), also improves lipids and macrovascular disease in diabetes (6). Notably, polyphenols are also widely reported to have beneficial effects on dyslipidemia in patients and animal models with diabetic cardiovascular disease (7,8), but no large trials have demonstrated their efficacy, and their mechanism(s) of action remains a mystery, limiting their therapeutic potential.

Searching for a signaling mechanism of the action of polyphenols, we tested the effects of polyphenols on AMPK activity in human HepG2 hepatocytes. AMPK is a key metabolic regulator in liver, skeletal muscle, and heart that responds to increased cellular AMP-to-ATP ratio and upstream signaling pathways stimulated by cellular stress (9). In turn, AMPK regulates fatty acid oxidation and lipid synthesis, two important determinants of tissue lipids and hyperlipidemia in diabetes (10). Previously, we showed that mimicking hyperglycemia by exposing HepG2 cells to high glucose inhibited phosphorylation of AMPK, decreased phosphorylation of the AMPK downstream target acetyl-CoA carboxylase (ACC), thus increasing its activity (11), and induced hepatocellular lipid accumulation (4). The inhibition of AMPK by high glucose was implicated causally in the lipid accumulation because the effects were mimicked by overexpression of an AMPK dominant-negative mutant. Conversely, the known AMPK activator metformin, or overexpression of an AMPK constitutively active mutant, increased phosphorylation of ACC and effectively prevented the accumulation of lipids caused by high glucose in HepG2 cells (4).

In a previous study, we found that a synthetic polyphenol, S17834, inhibited endothelial cell adhesion molecule expression, vascular oxidants, and atherogenesis in nondiabetic apolipoprotein (apo) E–deficient mice (12). We report here that S17834 decreases atherosclerosis in nondiabetic, LDL receptor–deficient (LDLR$^{-/-}$) mice. Because diabetes greatly enhances atherogenesis, we also

tested and found that S17834 prevented the accelerated atherogenesis in streptozotocin (STZ)-induced type 1 diabetic LDLR$^{-/-}$ mice. We noted that S17834 improved diabetic hyperlipidemia and that the ability of S17834 to prevent the acceleration of atherosclerosis by diabetes could be explained based on its ability to lower serum and hepatic lipids.

We report here that S17834 strongly and persistently stimulates AMPK phosphorylation and activity in HepG2 cells at concentrations 50–200 times lower than 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) or metformin. As a consequence, S17834 prevents cellular lipid accumulation caused by high glucose via an AMPK-dependent mechanism. Other polyphenols that are structurally similar to S17834 and known to have beneficial effects on hyperlipidemia, including resveratrol (a key component in red wine) and apigenin, had similar but less potent effects on hepatic AMPK activity and lipids. Hyperglycemia in diabetic LDLR$^{-/-}$ mice also decreased phosphorylation of AMPK and ACC in the liver, elevated hepatic and serum lipids, and accelerated aortic atherosclerosis. Treatment with S17834 augmented hepatic AMPK and ACC phosphorylation and thereby decreased hepatic and serum lipids, suppressing acceleration of atherosclerosis caused by diabetes. These studies identify AMPK activation as a novel molecular mechanism of action for polyphenols, like S17834 and resveratrol, to counter the effect of diabetic milieu on hyperlipidemia and accelerated atherogenesis.

RESEARCH DESIGN AND METHODS

S17834 [6,8-diallyl 5,7-dihydroxy 2-(2-allyl 3-hydroxy 4-methoxyphenyl)1-H benzo(b)pyran-4–one], a synthetic polyphenol, was obtained from the Institut de Recherches Servier (Suresnes, France). Apigenin and resveratrol were from Calbiochem (San Diego, CA). STZ, metformin (1,1-dimethylbiguanide), insulin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), AMP, Nonidet P-40, aprotinin, leupeptin, and phenylmethylsulfonyl fluoride were purchased from Sigma (St. Louis, MO). AICAR was from Toronto Research Chemicals (Downsview, ON, Canada). Fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM), and Dulbecco's PBS were from Gibco-BRL (Grand Island, NY). Rabbit polyclonal pan-AMPKα antibody and phospho-AMPKα (Thr-172) antibody were purchased from Cell Signaling Technology (Beverly, MA). Rabbit polyclonal anti-AMPKα subunit antibodies recognizing the α1 or α2 isoform as well as AMPKα1 and -α2 blocking peptides (the peptide sequences used to generate and immunopurify the antibodies) were from Bethyl Laboratories (Montgomery, TX). Rabbit polyclonal anti–phospho-Ser-79 ACC1 (Ser-221 ACC2) antibody, SAMS peptide, and P81 phosphocellular paper were purchased from Upstate Biotechnology (Lake Placid, NY). Mouse monoclonal anti-*myc* antibody (9E10) was from BD Biosciences (Palo Alto, CA). Mouse monoclonal anti–β-actin antibody was from Abcam (Cambridge, MA). Horseradish peroxidase–conjugated anti-mouse and anti-rabbit secondary antibodies and protein A/G plus agarose were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Adenopure kits for adenovirus purification were purchased from Puresyn (Malvern, PA). Enzymatic lipid assay kits (Infinity triglycerol and cholesterol reagents) were from Thermo DMA (Louisville, CO). ATPLite for measuring intracellular ATP and [γ-^{32}P]ATP were from PerkinElmer Life and Analytical Sciences (Boston, MA). All other reagents were of analytical grade.

Cell culture and treatments. The cultured human hepatoma HepG2 cell line was grown in DMEM containing normal glucose (5.5 mmol/l D-glucose) supplemented with 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin as previously described (4). Cells were incubated in a humidified atmosphere of 5% CO_2 at 37°C.

HepG2 cells were cultured in complete medium with 10% FBS to ~80% cell confluence and subjected to assays after overnight serum depletion. Polyphenols, including S17834, resveratrol, and apigenin, that were dissolved in DMSO were added to the medium. The final concentration of DMSO did not exceed 0.1%, which did not affect cell viability or AMPK phosphorylation. A cell model for high-glucose–induced accumulation of hepatic lipids was used by exposing HepG2 cells to a high concentration of glucose (30 mmol/l) for 24 h as described previously (4). Briefly, HepG2 cells were quiesced in serum-free DMEM overnight and incubated in DMEM containing either a normal (5.5 mmol/l) or high (30 mmol/l) concentration of D-glucose. The designation "normal glucose" refers to medium containing 5.5 mmol/l D-glucose, and "high glucose" refers to medium supplemented with 30 mmol/l D-glucose.

For the luminescence ATP detection assay, HepG2 cells (2.0×10^4 per well) were cultured in 96-well microplates and treated with polyphenols as indicated. Intracellular ATP levels were measured using ATPLite, an ATP monitoring system based on firefly (*Photinus pyralis*) luciferase, according to the manufacturer's instructions. A LumiCount microplate reader (SPECTR Max Gemini) was used to measure the luminescence as previously described (13).

For cellular toxicity, the MTT assay was performed according to the manufacturer's protocol (Sigma). HepG2 cells were seeded on a 96-well plate and grown to 70% confluence. Cells were treated for 24 h with increasing concentrations of S17834 in 100 μl of DMEM without phenol red and serum in quadruplicate for each condition, and they were subsequently incubated with 10 μl of the MTT solution (5 mg/ml in PBS) at 37°C for another 3 h, followed by incubation in 100 μl of 10% Triton X-100 and 0.1 N HCl in isopropanol for 10 min. The optical density at 570 nm was measured using a plate reader (SPECTRAmax340 microplate spectrophotometer; Molecular Devices). Cell viability was calculated from the optical density readings of S17834 treatment, using control cells as 100%. Treatment with S17834 (2.5–25 μmol/l) had no detectable effect on cell viability (data not shown).

Adenoviral infection. The recombinant adenoviral vector expressing a *myc*-tagged constitutively active mutant of AMPKα1 (Ad-CA-AMPK) that encodes residues 1–312 of AMPK α1 in which Thr-172 was substituted with aspartic acid [α1 (1–312) T172D] was generated as previously described (4,14). The adenoviral vector encoding a *myc*-tagged dominant-negative form of AMPKα2 (Ad-DN-AMPK), in which Lys-45 is substituted with arginine (α2K45R), was described previously (4,15). An adenoviral vector encoding green fluorescent protein (GFP; Ad-GFP) was used as a control. The adenoviruses were amplified in human embryonic kidney 293 cells that were cultured in DMEM supplemented with 10% FBS, and they were purified by Adenopure kits according to the manufacturer's instructions. The number of viral particles was estimated by measuring the optical density at 260 nm. In some experiments, HepG2 cells were transfected for 24 h in serum-free DMEM at 30–100 plaque-forming units of adenoviral vectors before treatment. Under these conditions, transfection efficiency was >80%, as determined by GFP expression.

Animal protocols and diets. Male homozygous LDL receptor–deficient (LDLR$^{-/-}$) mice (6 weeks of age) with C57BL/6 genetic background were created by homologous recombination (Jackson Laboratory, Bar Harbor, ME) (16). The mice were maintained on normal mouse chow and given free access to both food and water in a temperature- and light-controlled animal facility with a light/dark cycle of 6 A.M. to 6 P.M. After 1 week of acclimatization, diabetes was induced by intraperitoneal injection of STZ (100 mg · kg^{-1} · day^{-1}) dissolved in citrate buffer (0.05 mol/l, pH 4.5) for 5 consecutive days. Nondiabetic mice were injected with a comparable volume of citrate buffer. Glucose levels were measured in tail blood by a FreeStyle blood glucose monitoring system (TheraSense, Alameda, CA). Hyperglycemia was confirmed by nonfasting blood glucose >200 mg/dl (11 mmol/l) 1 week after initial STZ injection. The nondiabetic and diabetic LDLR$^{-/-}$ mice were randomly divided into two groups: untreated and S17834-treated mice. The untreated mice were fed normal mouse chow containing 4.5% fat. For S17834 treatment, customized chow diet of the same composition (Pharma Serv, Framingham, MA) was fed to mice and contained S17834 in sufficient amounts to administer a dose of 130 mg · kg^{-1} · day^{-1}. The dose of drug was calculated based on the average consumption of food (5 g/day) by a 23-g mouse. After 6 weeks the mice were killed under isoflurane anesthesia, and tissues were taken and frozen immediately in liquid nitrogen or fixed. AMPK phosphorylation was not different in liver harvested by freeze-clamping compared with liver that was quickly frozen in liquid nitrogen. Blood samples for serum lipids were collected from the vena cava. The Boston University Medical Center institutional animal care and use committee approved the protocol.

Assessment of aortic atherosclerosis. The whole aortas were collected and stained by oil red O (60% solubilized in propylene glycol) to detect the lipids present in lesions, as previously described (12). The entire aortic intimal surface was photographed and scanned digitally, and planimetry of oil red O–positive stained lesions was performed on the digitized images using Scion Image software. Quantification of atherosclerotic lesion area was expressed as the total aortic surface area covered by oil red O–positive lesions in square micrometers.

Immunoblotting analysis. Immunoblotting analysis was conducted as described previously (4,17,18). HepG2 cells were washed with PBS and lysed at 4°C in lysis buffer (20 mmol/l Tris-HCl, pH 8.0, 1% [vol/vol] Nonidet P-40, 1 mmol/l EDTA, 1 mmol/l EGTA, 1 mmol/l sodium orthovanadate, 1 mmol/l dithiothreitol, 1 mmol/l phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, 2

μg/ml leupeptin, and 1 μg/ml pepstatin), followed by centrifugation at 14,000 rpm for 10 min at 4°C. Protein concentrations in cell lysates were measured using a Bio-Rad protein assay kit. The cell lysates (20–50 μg protein) were combined with the appropriate amount of 6 × SDS sample buffer (0.32 mol/l Tris-HCl, pH 6.8, 30% [vol/vol] glycerol, 12% [wt/vol] SDS, 5% [vol/vol] β-mercaptoethanol, and bromophenol blue) and then heated at 95°C for 5 min. Samples were subjected to 8% SDS-PAGE and electrophoretically transferred to polyvinylidene difluoride membranes by wet transfer at 30 V overnight. The membranes were blocked with 5% (wt/vol) nonfat dry milk in Tris-buffered saline with Tween (TBST) buffer (20 mmol/l Tris-HCl, pH 7.6, 0.138 mol/l NaCl, and 0.1% [vol/vol] Tween 20) and subsequently blotted with the appropriate antibodies in TBST containing 1% (wt/vol) BSA. Antibodies were used at the following conditions: anti–phospho-AMPK (1:500 dilution) overnight at 4°C and anti–phospho-ACC antibody (1:5,000), anti–AMPKα1 or α2 antibodies (1:4,000), anti-*myc* antibodies (1:1,000), and anti–β-actin antibody (1:5,000) for 2 h at room temperature. The membranes were incubated with the secondary antibodies at a 1:10,000 dilution in TBST containing 5% (wt/vol) nonfat dry milk for 1 h, and the bound antibodies were visualized by an enhanced chemiluminescence system. Phosphorylated AMPK was quantified using a GS-700 Imaging Densitometer (Bio-Rad) and normalized to the levels of endogenous AMPKα protein. Unless stated otherwise, phosphorylation of ACC was expressed as the ratio of the sum of ACC1 and ACC2 phosphorylation to the level of endogenous AMPKα expression. In some cases, ACC1 and ACC2 bands were individually assessed by densitometry. Phosphorylation intensity of AMPK and ACC was expressed relative to the basal or control level.

Immunoprecipitation and kinase activity of AMPK. AMPK activity was measured after immunoprecipitation with polyclonal rabbit AMPKα1 or -α2 antibodies raised against synthetic peptides of AMPKα. For immunoprecipitation, as previously described (17,18), 200 μg of protein from HepG2 cell lysates was incubated with 25 μl of protein A/G plus agarose as well as isoform-specific AMPKα antibody or the antibody that was preincubated with competing peptide at 4°C overnight. The immunoprecipitates were washed once with lysis buffer, twice with lysis buffer containing 0.5 mol/l NaCl, and twice with kinase buffer (50 mmol/l HEPES, 80 mmol/l NaCl, and 1 mmol/l dithiothreitol, pH 7.4). AMPK activity in α1 or α2 immunocomplexes was measured using the SAMS peptide phosphorylation assay as described previously (19). Briefly, the kinase assay was performed in 40 μl kinase buffer containing 8 μl SAMS peptide (2.2 mg/ml), 200 μmol/l AMP, 5 mmol/l $MgCl_2$, 100 μmol/l ATP, and 10 μCi [γ-^{32}P]ATP (specific activity 2,000 cpm/pmol) at 30°C for 15 min with continuous shaking. Aliquots of the reaction mixture were spotted on 2 cm × 2 cm squares of P81 phosphocellular paper. The P81 paper was washed three times for 15 min in 0.38% (vol/vol) phosphoric acid with gentle stirring to remove free ATP and then dried to quantify ^{32}P-labeled incorporation into SAMS peptide by scintillation counting. Control immunoprecipitation that contained no AMPK antibody was performed to ensure that SAMS peptide phosphorylation was dependent on specific immunoprecipitation of AMPK. AMPK activity was calculated as picomoles per minute per milligram of protein.

Determination of cholesterol and triglyceride levels. Intracellular triglyceride and total cholesterol contents were measured in HepG2 cell lysates and expressed as micrograms of lipid per milligram of cellular protein, as previously described (4,20,21). Serum cholesterol and triglycerides were measured enzymatically, using Infinity reagents from Thermo DMA according to the manufacturer's instructions. Oil red O staining of fixed liver for lipid deposition was performed using established methods (22). We homogenized ~100 mg of liver tissue in 1 ml of PBS and determined the protein concentration. Homogenates were extracted with 5 ml of chloroform and methanol (2:1, vol/vol). The mixture was vortexed vigorously, allowed to separate into two phases, and centrifuged at 2,000 rpm for 10 min at 4°C. An aliquot of the organic phase was evaporated until dry under nitrogen gas. Hepatic total cholesterol and triglyceride concentrations were determined and normalized to protein concentrations, and they were expressed as milligrams of lipid per gram of tissue protein (23).

Statistical analyses. Data are the means ± SE. Statistical analysis was performed by a two-tailed unpaired Student's t test. $P < 0.05$ was considered to be statistically significant.

RESULTS

Polyphenols stimulate AMPK and ACC phosphorylation in cultured human HepG2 hepatocytes. Polyphenols have been shown to have beneficial effects on dyslipidemia in patients with diabetic cardiovascular diseases (7,8). Our recent studies demonstrated that the ability of metformin to lower lipid contents in cultured HepG2 cells was attributable to its ability to stimulate AMPK activity (4). Because our preliminary studies found that like metformin (2 mmol/l), S17834 (5–10 μmol/l), a synthetic polyphenol, also decreased lipid content in these cells at 24 h (see below and data not shown), we therefore tested whether S17834 activates AMPK in these cells. Because Thr-172 phosphorylation of the activation loop of the AMPKα catalytic domain is essential for activation of both the α1 and α2 subunits of AMPK (24,25), the activation state of total AMPKα was assessed by determining phosphorylation of AMPKα and its best-characterized downstream substrate, ACC, using immunoblots with specific phospho-Thr-172 and phospho-Ser-79 ACC1 (Ser-221 ACC2) antibodies as described previously (26–28). AMPK phosphorylates and inactivates ACC1 and ACC2 (29), which in turn downregulates lipid biosynthesis and upregulates fatty acid oxidation (30). Figure 1 shows the effect of treating HepG2 cells for 1 h with either known activators of AMPK, AICAR (1 mmol/l) and metformin (2 mmol/l), or S17834 (10 μmol/l), respectively. AICAR and metformin significantly stimulated AMPKα phosphorylation by 2.4- and 1.9-fold over the basal level, respectively (Fig. 1*A* and *B*). Importantly, S17834 at a concentration of 10 μmol/l caused a 3.6-fold increase in AMPKα phosphorylation. No change in the expression of endogenous AMPKα protein was noted by immunoblotting with AMPKα1 and -α2 antibodies (Fig. 1*A*). AMPK activation by S17834 (10 μmol/l) was further confirmed by enhanced phosphorylation of both ACC1 and ACC2 in HepG2 cells comparable to that of AICAR (1 mmol/l) and metformin (2 mmol/l) (Fig. 1*A* and *B*) and similar to that caused by adiponectin in cultured primary hepatocytes (31). Furthermore, concentrations as low as 2.5 μmol/l S17834 were found to significantly increase AMPK and ACC phosphorylation by twofold in 1 h (supplemental Fig. 1*A* and *B*, which is detailed in the online appendix [available at http://diabetes.diabetesjournals.org]), reaching levels that were similar to those induced by AICAR (1 mmol/l) or metformin (2 mmol/l) in 1 h (Fig. 1*A* and *B*). Three- to fourfold stimulation of phospho-AMPK and phospho-ACC occurred at higher concentrations of S17834 (10–25 μmol/l) (supplemental Fig. 1*A* and *B*). Increased phosphorylation level of AMPK caused by S17834 closely correlated with the increase in ACC phosphorylation. These results indicate that S17834 stimulates AMPK phosphorylation and downstream activity in a dose-dependent manner. Moreover, the phosphorylation of AMPK and ACC occurred very rapidly, rising to near maximal levels within 10 min, and was sustained for 24 h (Fig. 1*C*).

To determine whether other polyphenols that are structurally similar to S17834 (Fig. 1*D*) and have previously been shown to lower lipids have a stimulatory effect on AMPK, the ability of apigenin and resveratrol to activate AMPK was studied in HepG2 cells. When cells were maintained in normal glucose, apigenin (10 μmol/l, 24 h) caused a slight but statistically insignificant increase in AMPK and ACC phosphorylation. Resveratrol (10 μmol/l), like S17834, significantly increased AMPK and ACC phosphorylation, but to a lesser extent (Fig. 1*E* and *F*).

To further confirm that the polyphenols stimulate AMPK activity, AMPKα1 or -α2 immunoprecipitates prepared from HepG2 cells were assayed for AMPK kinase activity using SAMS peptide as a substrate. As shown in Fig. 2*A*, a band (molecular weight 63 kDa) representing the AMPKα1 or -α2 isoform in immunoprecipitates is detected by immunoblotting with the corresponding AMPKα isoform

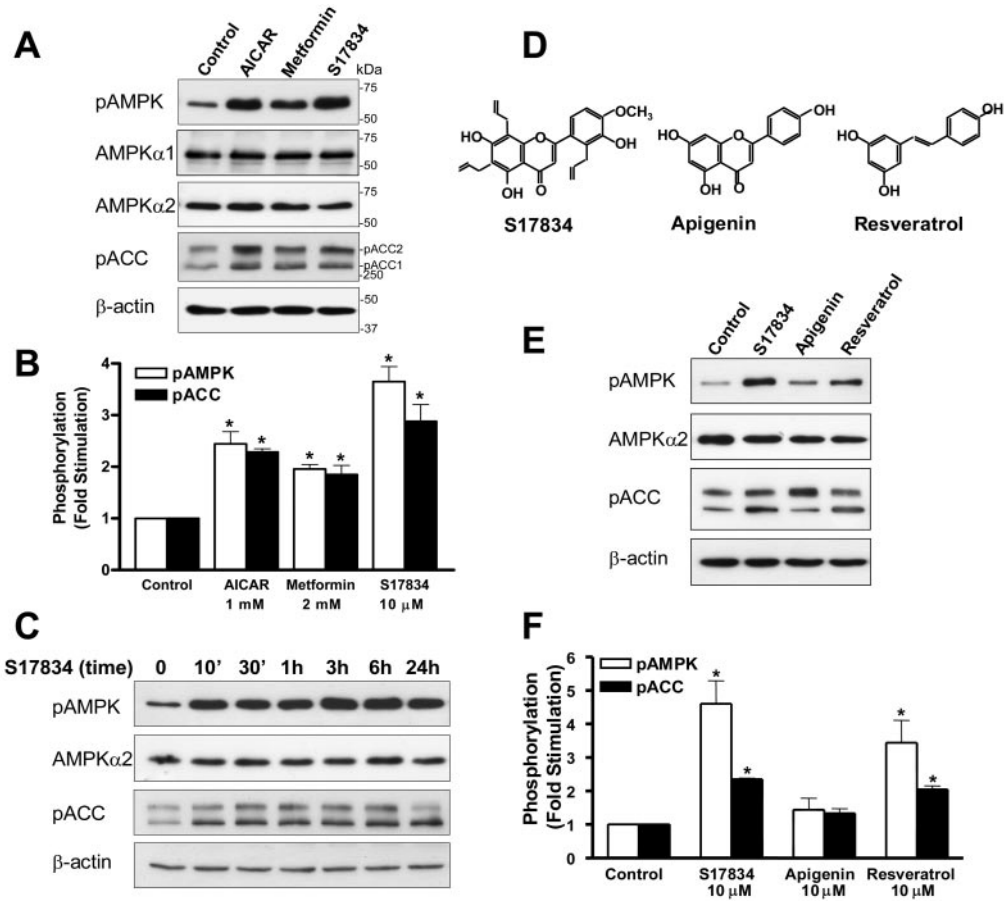

FIG. 1. Polyphenols increase phosphorylation of both AMPK and ACC in cultured human HepG2 hepatocytes. *A* and *B*: S17834 (10 μmol/l) mimics the effect of AICAR (1 mmol/l) and metformin (2 mmol/l) on AMPK and ACC phosphorylation. HepG2 cells were quiesced in serum-free DMEM overnight and treated with AICAR, metformin, or S17834 for an additional 1 h. Cell lysates (50 μg proteins) were resolved by 8% SDS-PAGE as described under RESEARCH DESIGN AND METHODS. The phosphorylation of AMPK and its downstream target, ACC, was assessed by using immunoblots with phospho-Thr-172 AMPKα (pAMPK) and phospho-Ser-79 ACC1 (Ser221 ACC2; pACC) antibodies. The levels of phosphorylated AMPK and the sum of phosphorylated ACC1 and ACC2 were normalized to endogenous AMPKα levels and expressed as the fold stimulation over the basal level (means ± SE, $n = 3$). *C*: Time course effect of S17834 on AMPK and ACC phosphorylation. The phosphorylation of AMPK and ACC is shown in HepG2 cells exposed to S17834 (10 μmol/l) for 10 and 30 min and 1, 3, 6, and 24 h. *D*: Chemical structures of polyphenols. *E* and *F*: Effect of apigenin and resveratrol on AMPK and ACC phosphorylation. A representative immunoblot and densitometric quantification of the phosphorylation of AMPK and ACC in HepG2 cells incubated with 10 μmol/l of polyphenols for 24 h are shown. *$P < 0.05$ compared with vehicle control.

antibody or with anti–AMPK pan-α antibody. The immunoreactive band was eliminated by preincubation with AMPKα1 or -α2 immungenic peptides, indicating the specificity of the α1 and α2 AMPK isoform antibodies. AMPKα1 has been observed to be the predominant isoform in liver or hepatocytes (14,31). Therefore, the basal activity of the AMPKα1 isoform in HepG2 cells was observed to be 10-fold higher than that of AMPKα2. S17834 (10 μmol/l) or resveratrol (50 μmol/l) for 1 h significantly enhanced AMPKα1 activity, similar to that induced by the AMPK activator metformin (2 mmol/l). Although the basal activity of AMPK was lower, S17834 caused a fivefold increase in AMPKα2 activity, which was greater than the increase caused by resveratrol or metformin. The AMPKα1 and -α2 activity induced by polyphenols or metformin was completely blocked when the isoform-specific immunoprecipitating antibodies were preincubated with the respective blocking peptides (Fig. 2*B* and *C*). The results indicated that both AMPKα1 and -α2 isoforms contribute to AMPK activation in response to polyphenols.

To elucidate the mechanisms responsible for polyphenol-induced activation of AMPKα1 and -α2, we determined whether polyphenols decrease the concentration of cellular ATP, which would result in an increase in the AMP-to-ATP ratio. After treating cells with S17834 (10 μmol/l), no change in ATP level was evident up to 30 min, but there was a significant decrease at 1 h (Fig. 2*D*). Treatment with S17834 (5 μmol/l) or resveratrol (10 μmol/l) for 1 h had no effect on ATP levels (Fig. 2*E*). This indicates that the rapid activation of AMPK by S17834 within 30 min is likely to be independent of ATP hydrolysis, although persistent activation of AMPK by polyphenols at higher concentrations may result from changes in adenine nucleotide concentration.

High-glucose–induced lipid accumulation is prevented by polyphenols in HepG2 cells. We previously reported that exposing HepG2 to elevated glucose (30 mmol/l) for 24 h decreases AMPK and ACC phosphorylation and induces hepatocellular lipid accumulation (4). These effects of high glucose are under the control of AMPK, which is attenuated or augmented, respectively, by transfection with AMPK constitutively active or dominant-negative adenoviral vectors (4). In the current study, we found that the inhibition of AMPK and ACC phosphorylation occurred very rapidly at 1 h after exposing the cells to high glucose, reducing the levels to ~50% of control, and was sustained up to 24 h (Fig. 3*A* and *B*). Stimulation of AMPK and ACC phosphorylation by preincubation with S17834 (10 μmol/l, 1 h) countered the decrease in AMPK and ACC phosphorylation caused by high glucose and maintained their levels at the same levels as those observed in cells incubated in normal glucose throughout 24 h (Fig. 3*A* and *B*). Moreover, exposure of HepG2 cells to high glucose for 24 h decreased AMPKα1 activity by 50%, without detectable changes in AMPKα2 activity, and S17834 prevented the inhibition of AMPKα1 more effectively than metformin (Fig. 4*C*).

Intracellular levels of triglycerides were increased in HepG2 cells as early as 6 h after incubation in high glucose (Fig. 3*C*). S17834 (10 μmol/l) largely prevented the increase in hepatocellular triglycerides at 6 and 24 h, and it was as effective as metformin (2 mmol/l) (4). S17834 (10 μmol/l) also decreased the cholesterol content of HepG2 cells incubated in high glucose for 6 or 24 h (Fig. 3*D*), and

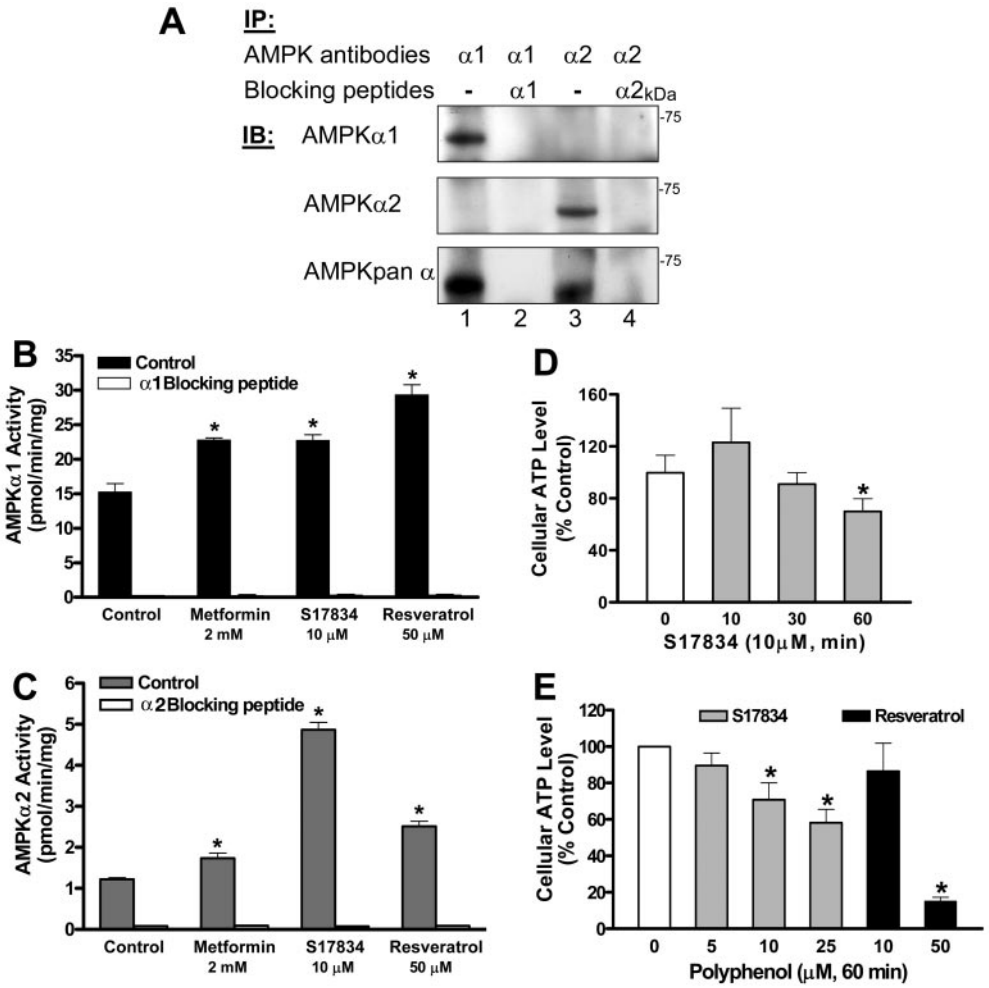

FIG. 2. Polyphenols stimulate isoform-specific AMPK-α activity in HepG2 cells. *A*: Specificity of immunoprecipitation (IP) of the α-isoforms of AMPK. HepG2 cell proteins were immunoprecipitated with either AMPKα1 antibody (*lanes 1* and *2*) or AMPKα2 antibody (*lanes 3* and *4*) or with the antibodies preincubated with blocking peptides of AMPKα1 (*lane 2*) or AMPKα2 (*lane 4*) as described under RESEARCH DESIGN AND METHODS. Immunoblotting (IB) was then conducted with AMPKα1, AMPKα2, and AMPK pan-α antibodies, respectively. Immunoprecipitation was AMPKα isoform–specific with no detectable cross-reactivity with immunoprecipitated AMPKα1 or -α2 proteins. *B* and *C*: Polyphenols increase both AMPKα1 and -α2 activity. HepG2 cells were quiesced in serum-free DMEM overnight and treated with metformin, S17834, or resveratrol for an additional 1 h. AMPK activity in α1 or α2 immunocomplexes in the absence and presence of α1 or α2 blocking peptide was measured using the SAMS peptide phosphorylation assay as described under RESEARCH DESIGN AND METHODS. AMPK activity was calculated as the picomoles per minute per milligram protein. Data are the means ± SE (n = 4). *P < 0.05 compared with vehicle control. *D* and *E*: Effect of polyphenols on intracellular ATP levels. The ATP concentrations in HepG2 cells treated with S17834 or resveratrol as indicated were measured by a luciferase-based assay and expressed as a percentage of that under control conditions. Data are the means ± SE (n = 4). *P < 0.05 compared with vehicle control.

the effect at 24 h was as great as that of metformin (2 mmol/l) (4).

To determine whether two other polyphenols have similar actions as S17834, the ability of apigenin and resveratrol to activate AMPK and inhibit hepatocellular lipids was studied in HepG2 cells exposed to elevated glucose. Apigenin (10 μmol/l) or resveratrol (10 μmol/l) for 24 h significantly stimulated AMPK and ACC phosphorylation (Fig. 4*A* and *B*) as well as AMPKα1 activity, but not AMPKα2 activity (Fig. 4*C*), above the levels of untreated cells incubated in elevated glucose. In addition, both polyphenols inhibited high-glucose–induced accumulation of triglycerides and cholesterol similarly to S17834 (Fig. 4*D*). Because of the stronger effect of S17834 on AMPKα activity and ACC phosphorylation, S17834 was used in all subsequent studies.

The effect of S17834 on high-glucose–induced lipid accumulation in HepG2 cells is mimicked by overexpression of an AMPK constitutively active mutant. To determine the role of AMPK in mediating the lipid-lowering effects of these polyphenols in HepG2 cells exposed to elevated glucose, the effects of S17834 were compared with those of transfection with a *myc*-tagged constitutively active AMPK mutant [α1 (1–312) T172D] (4,14). This truncation mutant of AMPKα1 retains significant kinase activity without requiring association with β- and γ-subunits, and mutation of Thr-172 to aspartic acid within the truncated α-subunit mimics the effect of phosphorylation and therefore functions as a constitutively active enzyme (14). We previously showed that overexpression of the constitutively active AMPK mutant prevented the decrease in AMPK and ACC phosphorylation caused by high glucose and was as effective as metformin in preventing the accumulation of lipids in HepG2 cells exposed to elevated glucose (4). The current studies confirmed these findings and showed that AMPK and ACC phosphorylation was similar in cells exposed to normal glucose and cells exposed to elevated glucose and either treated with S17834 (10 μmol/l) or transfected with the constitutively active AMPK mutant. Similar changes in phosphorylation of ACC1 or ACC2 were noted (Fig. 5*A* and *B*), consistent with both ACC1 and ACC2 as mediators of the effect of S17834 in regulation of fatty acid synthesis and oxidation. Furthermore, S17834 (10 μmol/l) or overexpression of the constitutively active AMPK mutant had similar effects in decreasing triglyceride levels in cells exposed to normal glucose or attenuating the increase caused by high glucose (Fig. 5*C*). In fact, there was no additive effect on triglyceride levels in HepG2 cells treated with the combination of S17834 and transfection with the constitutively active AMPK mutant, suggesting a similar mechanism of action. Likewise, S17834 or transfection with the constitutively active AMPK mutant similarly lowered cholesterol contents of HepG2 cells (Fig. 5*D*).

The effect of S17834 on high-glucose–induced lipid accumulation in HepG2 cells is prevented by overexpression of a dominant-negative AMPK mutant. To further determine the role of AMPK in the beneficial action

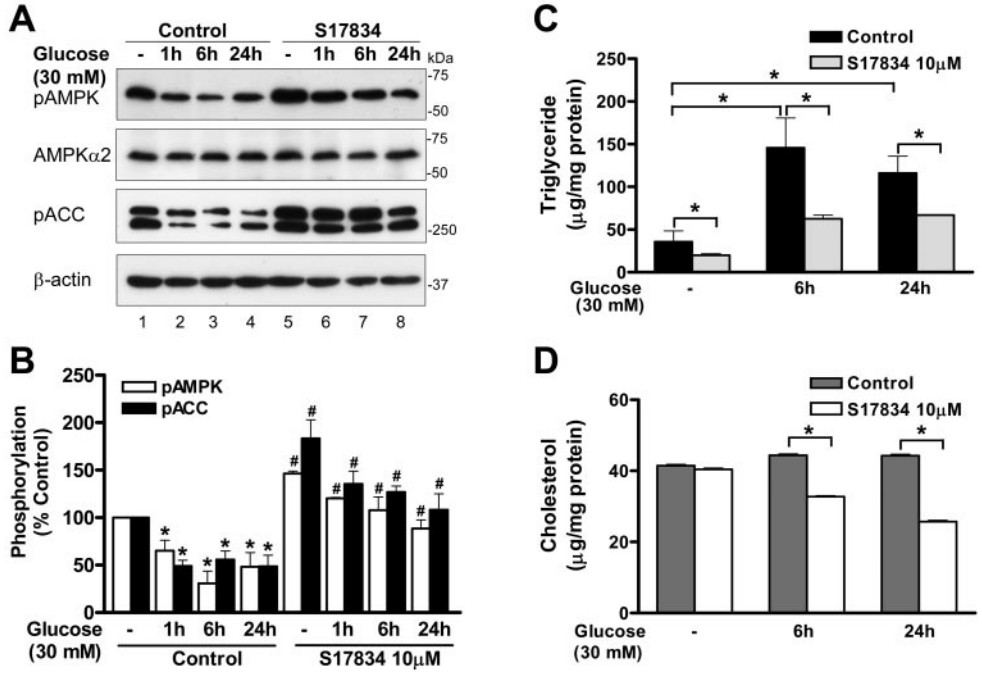

FIG. 3. S17834, a synthetic polyphenol, prevents the inhibition of AMPK and accumulation of lipids caused by high glucose in HepG2 cells. HepG2 cells quiesced in serum-free medium with normal glucose (5.5 mmol/l) were pretreated with DMSO (0.1%) or S17834 (10 μmol/l, 1 h), followed by incubation with high concentrations of glucose (30 mmol/l) alone or in combination with S17834 (10 μmol/l) for the indicated time. *A*: S17834 and high glucose reciprocally regulate AMPK signaling. Total cell extracts were immunoblotted for phospho-Thr-172 AMPKα and phospho-ACC1, as well as for AMPKα2 and β-actin as loading controls. *B*: The phosphorylation of AMPK and ACC was expressed as a percentage of that under control conditions. Data are the means ± SE (n = 3). *P < 0.05 compared with normal glucose control; #P < 0.05 compared with the high glucose alone at the same time points. *C* and *D*: S17834 lowers HepG2 cell lipid contents and protects against high-glucose–induced lipid accumulation. Intracellular triglyceride and cholesterol contents in cell lysates were expressed as micrograms lipid per milligram protein as described under RESEARCH DESIGN AND METHODS. Data are the means ± SE (n = 4). *P < 0.05 compared between two groups as indicated. pACC, phospho-ACC; pAMPK, phospho-AMPKα.

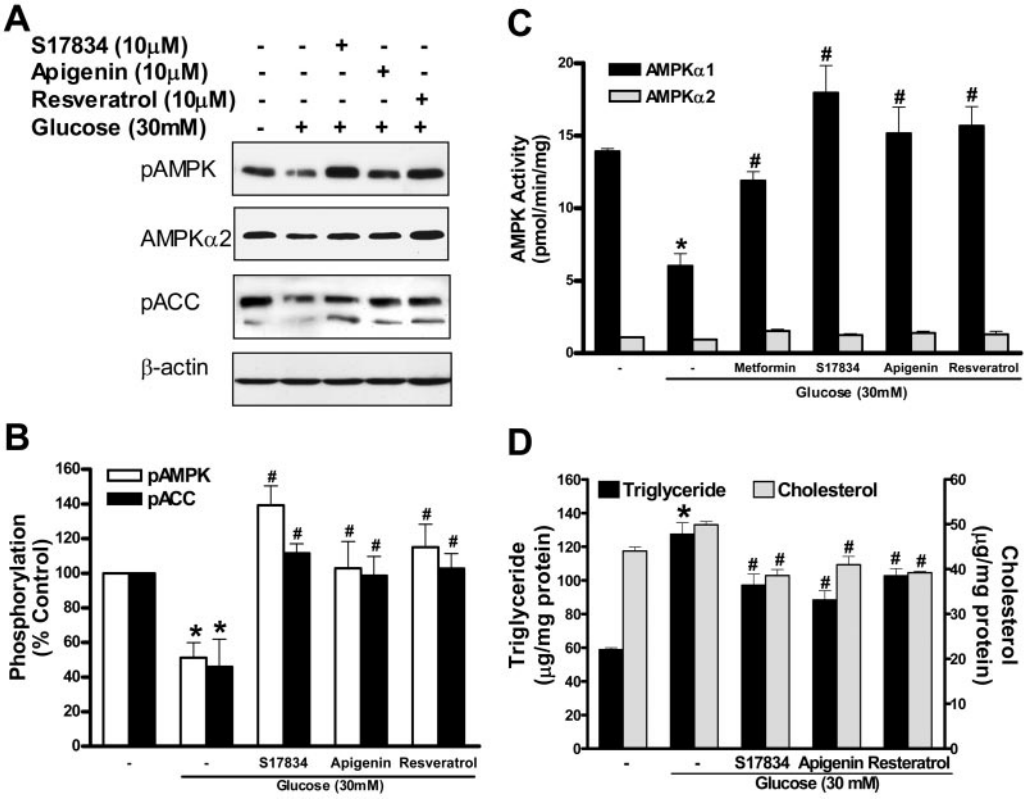

FIG. 4. Polyphenols stimulate AMPK phosphorylation and activation and inhibit lipid accumulation in HepG2 cells exposed to high glucose. HepG2 cells were maintained in serum-free medium overnight and incubated for 24 h with high glucose (30 mmol/l) alone or in combination with 10 μmol/l of polyphenols, including S17834, apigenin, and resveratrol. *A* and *B*: Polyphenols prevent inhibition of AMPK and ACC phosphorylation caused by high glucose. Representative immunoblots of phosphorylation of AMPK and ACC are shown. The phosphorylation levels of AMPK and ACC were expressed as a percentage of those in normal glucose exposed control cells. Data are the means ± SE (n = 3). *C*: Polyphenols prevent the ability of high glucose to inhibit AMPKα1 activity. The kinase activity of AMPKα1 and -α2 was measured and expressed as the means ± SE (n = 4). *D*: Polyphenols suppress high-glucose–induced lipid accumulation. Levels of intracellular triglycerides and cholesterol are represented as the means ± SE (n = 3). *P < 0.05 compared with normal glucose control; #P < 0.05 compared with high glucose alone. pACC, phospho-ACC; pAMPK, phospho-AMPKα.

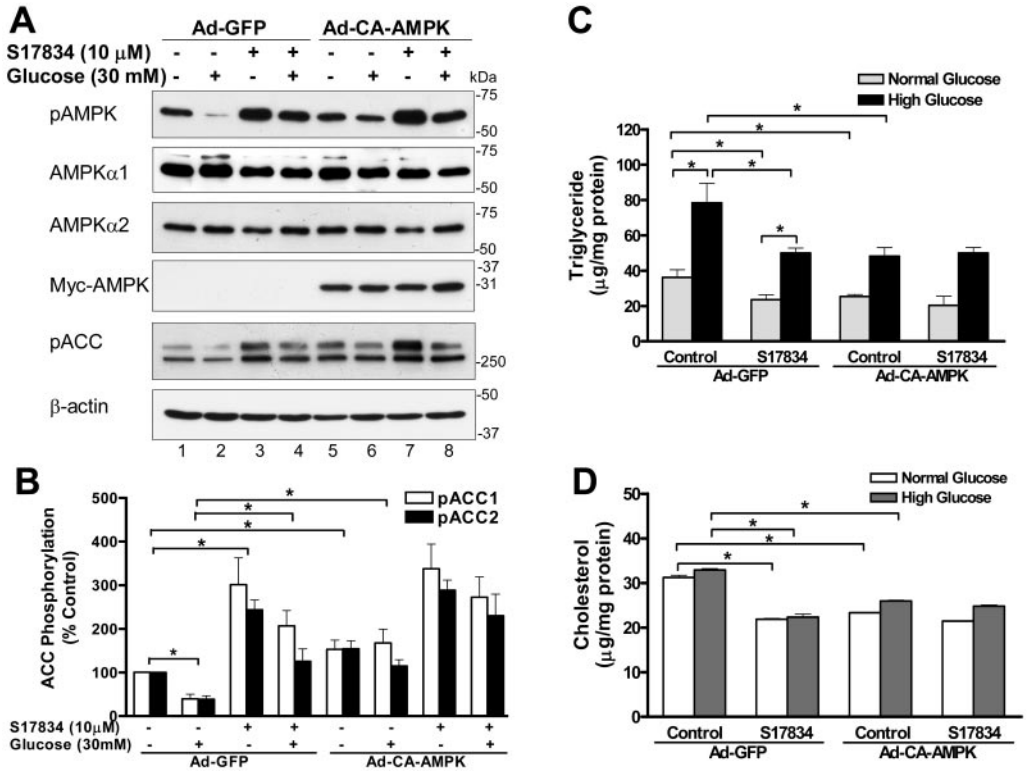

FIG. 5. Stimulation of AMPK and suppression of lipid accumulation by S17834 is mimicked by expression of a constitutively active form of AMPK in HepG2 cells. HepG2 cells were infected with adenoviral vectors encoding GFP (Ad-GFP) or a *myc*-tagged constitutively active form of AMPKα [Ad-CA-AMPK; α1 (1–312) T172D] in serum-free DMEM overnight before treatment with S17834 (10 μmol/l) in DMEM containing either normal glucose (5.5 mmol/l) or high glucose (30 mmol/l) for 24 h. *A* and *B*: Expressing the constitutively active AMPK is sufficient to prevent inhibition of AMPK by high glucose and mimic AMPK activation by S17834. Expressed recombinant constitutively active AMPK protein (~31 kDa) was detected by immunoblotting, using anti-*myc* antibody. Total cell extracts were immunoblotted for phosphorylation as well as for the expression of AMPKα1 and -α2 and β-actin. The phosphorylation levels of ACC1 or ACC2 determined separately by densitometry were normalized to endogenous AMPKα level and represented as the means ± SE (n = 4). *P < 0.05 compared between two groups as indicated. *C* and *D*: Overexpression of the constitutively active AMPK is sufficient to decrease lipid contents of HepG2 cells incubated in either normal or high glucose, similar to the effect of S17834. Levels of intracellular triglyceride and cholesterol are represented as the means ± SE (n = 4). *P < 0.05 compared between two groups as indicated. pACC, phospho-ACC; pAMPK, phospho-AMPKα.

of S17834 on hepatic lipid accumulation, HepG2 cells were transfected with a dominant-negative AMPK mutant that was shown to replace the endogenous α-subunits and suppress both AMPKα1 and -α2 activities (15). The dominant-negative AMPK mutant has previously been demonstrated to block the lipid-lowering action of metformin in these cells (4). Transfection with the dominant-negative AMPK mutant largely prevented the increase in ACC1 and ACC2 phosphorylation caused by S17834 (10 μmol/l) in cells exposed to normal or high glucose and transfected with a control vector that overexpressed GFP (Fig. 6*A* and *B*). Furthermore, overexpression of the dominant-negative AMPK mutant abrogated the ability of S17834 to decrease triglyceride levels, most notably in cells incubated in high glucose. Similarly, the ability of S17834 (10 μmol/l) to lower cholesterol levels was prevented by overexpression of the dominant-negative AMPK mutant in HepG2 cells incubated in high glucose (Fig. 6*C* and *D*). These results reveal that AMPK is necessary for the effect of S17834 to prevent accumulation of hepatocellular lipids caused by high glucose.

S17834 lowers serum and hepatic lipids in STZ-induced diabetic LDLR$^{-/-}$ mice in vivo. To test the effect of S17834 on dyslipidemia and atherogenesis in diabetes, type 1 diabetes was induced in LDLR$^{-/-}$ mice at 8 weeks of age by injection of STZ (100 mg · kg^{-1} · day^{-1} i.p.) for 5 days. Induction of diabetes was confirmed by levels of blood glucose >200 mg/dl (11 mmol/l) 1 week after STZ injection. At the end of the experiment (7 weeks after injection), blood glucose levels in STZ-injected mice were significantly higher than those in the control mice, and treatment with S17834 (130 mg · kg^{-1} · day^{-1}) had no effect on the level of hyperglycemia (supplemental Table, which is detailed in the online appendix). Even though the body weight and heart weight were significantly lower in STZ-treated mice, there was no significant change in heart weight–to–body weight ratio between control and STZ-injected mice. There was also no significant effect of treatment with S17834 on heart weight or body weight in either nondiabetic or diabetic LDLR$^{-/-}$ mice (supplemental Table).

To examine whether S17834 lowers serum and hepatic lipid levels in diabetic LDLR$^{-/-}$ mice, serum cholesterol levels were compared in control and S17834-treated nondiabetic and diabetic LDLR$^{-/-}$ mice. As previously found in nondiabetic apoE$^{-/-}$ mice (12), S17834 (130 mg · kg^{-1} · day^{-1}) had no significant effect on serum total cholesterol levels in nondiabetic LDLR$^{-/-}$ mice (Fig. 7*A*). As reported previously in STZ-induced diabetic apoE$^{-/-}$ mice (32), cholesterol levels were elevated by approximately threefold in STZ-induced diabetic LDLR$^{-/-}$ mice compared with nondiabetic LDLR$^{-/-}$ mice (Fig. 7*A*). Importantly, unlike in nondiabetic mice, S17834 strongly decreased serum cholesterol and triglyceride levels in diabetic LDLR$^{-/-}$ mice (Fig. 7*A*). Thus, S17834 was revealed to have bene-

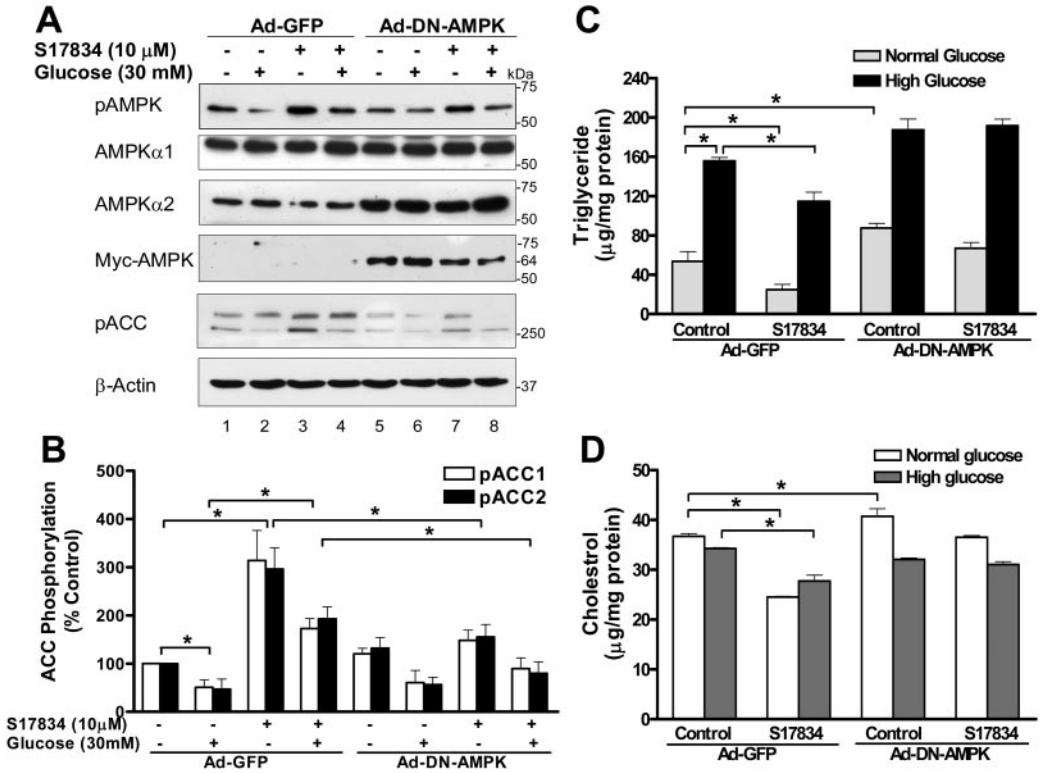

FIG. 6. Effect of S17834 on ACC1 and ACC2 phosphorylation and lipids in HepG2 cells is mediated by AMPK. Adenoviral vectors encoding a *myc*-tagged dominant-negative AMPK (Ad-DN-AMPK; AMPKαK45R) or Ad-GFP was used to infect into HepG2 cells in serum-free DMEM overnight. Cells were subsequently incubated with S17834 (10 μmol/l) in DMEM containing either normal glucose (5.5 mmol/l) or high glucose (30 mmol/l) for another 24 h. *A* and *B*: The stimulatory effect of S17834 on ACC phosphorylation is prevented by the dominant-negative AMPK. Expression of the dominant-negative AMPK recombinant protein (~64 kDa) was detected by immunoblotting for anti-*myc* and anti-AMPKα2 antibodies. The phosphorylation of AMPK and ACC was assessed by immunoblotting with phospho-Thr-172 AMPK or phospho-ACC (Ser-79 in ACC1 and Ser221 in ACC2) antibodies. Each bar represents the means ± SE ($n = 4$) of the ACC1 or ACC2 phosphorylation determined separately by densitometry. *$P < 0.05$ compared between two groups as indicated. *C* and *D*: Lipid-lowering action of S17834 was abrogated by overexpression of the dominant-negative AMPK. Levels of HepG2 cell triglyceride and cholesterol are expressed as the means ± SE ($n = 4$). *$P < 0.05$ compared between two groups as indicated. pACC, phospho-ACC; pAMPK, phospho-AMPKα.

ficial effects on serum cholesterol only in the setting of hyperglycemia associated with diabetes.

Furthermore, oil red O staining of liver sections showed that S17834 treatment dramatically decreased the accumulation of hepatic lipids in diabetic LDLR$^{-/-}$ mice (supplemental Fig. 2, which is detailed in the online appendix). Likewise, the content of triglycerides and cholesterol in the liver was increased more than twofold in the diabetic mice, and treatment with S17834 significantly lowered their levels by ~40% (Fig. 7*B*). These results indicate that S17834 prevents hepatic lipid accumulation, which is associated with decreased hyperlipidemia in diabetic LDLR$^{-/-}$ mice.

Diabetes inhibits and S17834 stimulates AMPK in the liver in vivo. Having obtained evidence that AMPK activation is required for the ability of S17834 to increase both ACC1 and ACC2 phosphorylation and to decrease lipid contents in HepG2 cells in vitro, we investigated possible mechanisms accounting for the lipid-lowering effect of S17834 in diabetic LDLR$^{-/-}$ mice in vivo. Compared with nondiabetic mice, Thr-172 phosphorylation of AMPK was dramatically decreased in diabetic LDLR$^{-/-}$ mouse livers by ~60% without a change in total AMPKα1 or -α2 expression (Fig. 7*C* and *D*). Similarly, inhibition of AMPK activity was confirmed by decreased phosphorylation of ACC in the livers of the diabetic mice (Fig. 7*C* and *D*). In diabetic LDLR$^{-/-}$ mice treated with S17834 (130 mg · kg^{-1} · day^{-1}), phosphorylation of AMPK and ACC was increased approximately twofold, again with no difference seen in the expression of AMPKα1 or -α2 or β-actin (Fig. 7*C*). These results indicate that hyperglycemia inhibits AMPK and ACC phosphorylation in the livers of diabetic LDLR$^{-/-}$ mice (Fig. 7*C* and *D*), mimicking the effects of high glucose on AMPK activity and phosphorylation in HepG2 cells in vitro (Figs. 3*B* and 4*C*). Furthermore, our results indicate that activation of AMPK by treatment with S17834 has effects on lipid accumulation in the liver in vivo that are similar to those observed in HepG2 cells, which require AMPK for the lipid-lowering effect of S17834.

S17834 inhibits the development of aortic atherosclerosis in STZ-induced diabetic LDLR$^{-/-}$ mice. Because polyphenols may have beneficial effects on dyslipidemia and its accelerated aortic atherosclerosis in diabetes, we examined whether the lipid-lowering effect of S17834 may attenuate atherosclerosis, which is accelerated in diabetic LDLR$^{-/-}$ mice. At the end of the 6-week treatment period, at 15 weeks of age, nondiabetic LDLR$^{-/-}$ mice had small atherosclerotic lesions in the aortic arch (Fig. 8*A*). Lesion area determined by computerized image analysis showed that these early lesions in nondiabetic LDLR$^{-/-}$ mice were significantly decreased by ~40% during treatment with S17834 (130 mg · kg^{-1} · day^{-1}) (Fig. 8*A* and *B*), similar to that observed in S17834-treated nondiabetic apoE$^{-/-}$ mice (12). STZ-induced diabetic mice showed dramatically larger lesions that were most extensive in the aortic arch, but they also included lesions at the

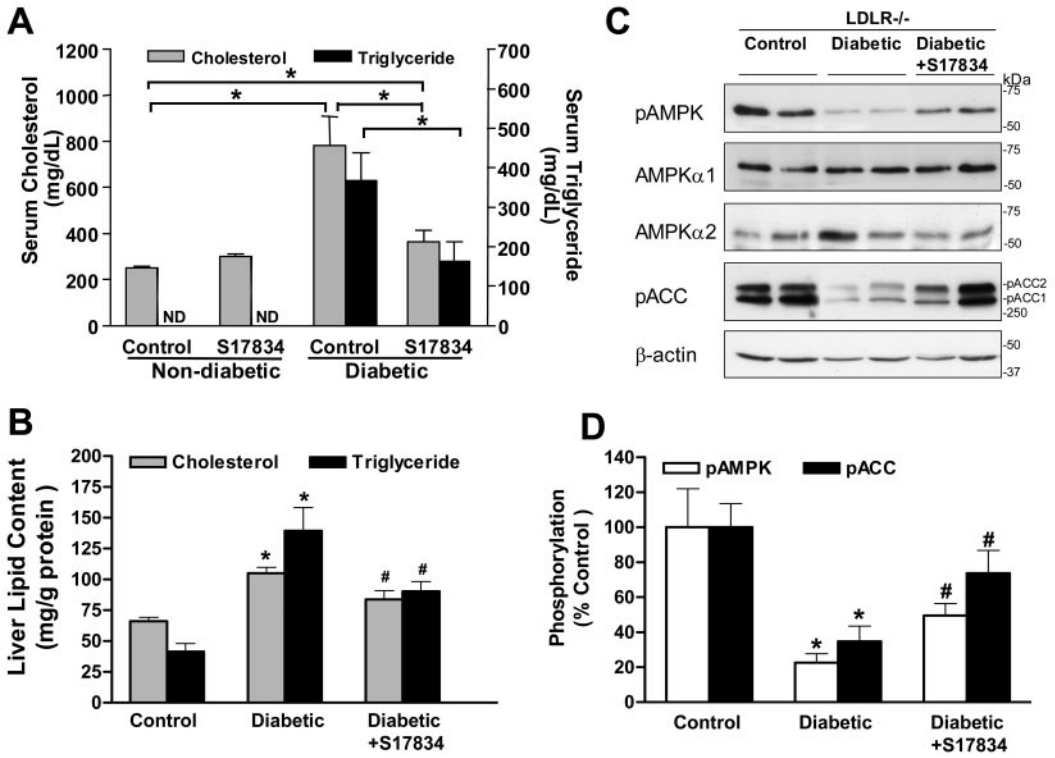

FIG. 7. Treatment with S17834 lowers serum and hepatic lipid levels as well as stimulates the phosphorylation of AMPK and ACC in the liver of STZ-induced diabetic LDLR$^{-/-}$ mice. *A*: S17834 reduces serum total cholesterol and triglyceride levels in diabetic LDLR$^{-/-}$ mice. Serum lipid levels were analyzed as described under RESEARCH DESIGN AND METHODS and expressed as the means ± SE (n = 15–25) in nondiabetic and diabetic LDLR$^{-/-}$ mice treated or not treated with S17834 (130 mg · kg^{-1} · day^{-1} for 6 weeks). *P < 0.05 compared between two groups as indicated. ND, not determined. *B*: S17834 decreases the lipid content in diabetic mouse livers. Liver lipids were extracted, and cholesterol and triglyceride levels were measured as described under RESEARCH DESIGN AND METHODS and expressed as milligrams of lipid per gram protein. Data are the means ± SE (n = 5–7). *C* and *D*: Phosphorylation of AMPK and ACC is suppressed in the livers of diabetic LDLR$^{-/-}$ mice, and this impairment is prevented by treatment with S17834. Equal amounts of liver protein extract (100 μg) were separated by 8% SDS-PAGE. A representative immunoblot of phosphorylation of AMPK and ACC and equal expression of total AMPKα1 or -α2 and β-actin in the livers from two mice in each group is shown. Quantitative analysis of phosphorylated AMPK and ACC is expressed as the means ± SE (n = 4) in the livers as described in Fig. 1. *P < 0.05 versus nondiabetic LDLR$^{-/-}$ mice; $^{\#}P$ < 0.05 versus untreated diabetic LDLR$^{-/-}$ mice. pACC, phospho-ACC; pAMPK, phospho-AMPKα.

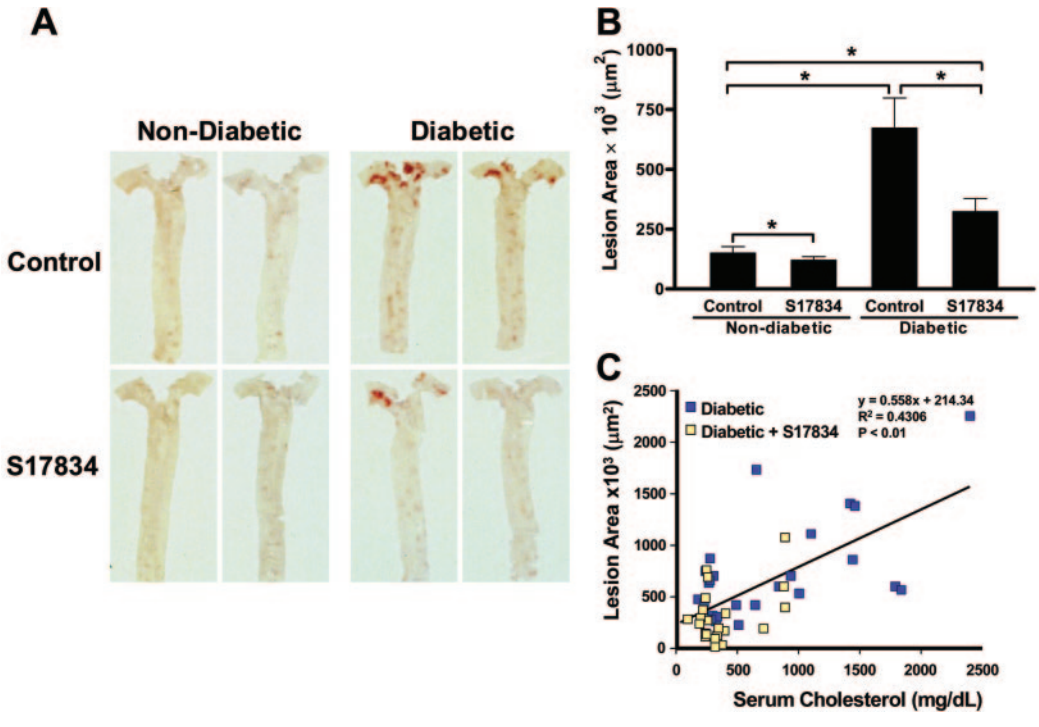

FIG. 8. Treatment with S17834 reduces aortic atherosclerotic lesions of STZ-induced diabetic LDLR$^{-/-}$ mice in large part through its lipid-lowering effect. *A*: S17834 attenuates aortic atherosclerotic lesions in diabetic LDLR$^{-/-}$ mice. A representative oil red O staining of aortic intima is shown in nondiabetic and diabetic LDLR$^{-/-}$ mice treated or not treated with S17834 (130 mg · kg^{-1} · day^{-1} for 6 weeks). *B*: Quantitative analysis of oil red O–stained atherosclerotic lesions was performed using computer-assisted image analysis. Bar graph represents the means ± SE (n = 10–16). *P < 0.05 as indicated. *C*: Serum cholesterol concentrations were significantly correlated with aortic atherosclerotic lesion area in individual diabetic LDLR$^{-/-}$ mice treated or not treated with S17834.

spinal artery branches (Fig. 8*A*). Mean aortic lesion area was increased by approximately fourfold in diabetic LDLR$^{-/-}$ mice (Fig. 8*B*). Treatment with S17834 throughout the 6-week period of diabetes resulted in a twofold reduction in lesion area (Fig. 8*A* and *B*). Notably, in untreated diabetic LDLR$^{-/-}$ mice and those treated with S17834, a statistically significant correlation existed between serum cholesterol levels and aortic atherosclerotic lesion area ($R^2 = 0.4306$, $P < 0.01$) (Fig. 8*C*). Because treatment with S17834 decreased atherosclerotic lesions to near the levels observed in nondiabetic LDLR$^{-/-}$ mice, these observations indicate that S17834 exhibits its beneficial effect on aortic atherosclerosis in diabetic LDLR$^{-/-}$ mice in large part through its lipid-lowering effect.

DISCUSSION

We have discovered that several polyphenols stimulate AMPKα1 and -α2 activity and ACC phosphorylation. The effect of polyphenols was 50–200 times more potent than that of AICAR or metformin. This action countered the inhibition of AMPKα1 activity and ACC phosphorylation as well as the increased hepatocellular lipid accumulation caused by elevated glucose, and it was demonstrated both in vitro and in vivo. The in vivo effect of one of the polyphenols, S17834, was associated with a large decrease in serum lipids to which could be attributed the prevention of the accelerated atherosclerosis in type 1 diabetic LDLR$^{-/-}$ mice. Our finding that structurally related polyphenols display comparable effects on hepatocellular lipids indicates that the activation of AMPK may account for the lipid-lowering actions of these and other polyphenols.

AMPK acts as a fuel sensor (33), so it is not surprising that in vivo or in vitro exposure to high glucose decreases hepatic AMPK phosphorylation and activity and its energy-conserving effects on downstream signaling targets. Intracerebral administration of high glucose also dephosphorylates and inactivates hypothalamic AMPK (24). Inhibition of AMPK was also observed in pancreatic cell lines exposed to elevated glucose (30 mmol/l) (34), in cultured hepatocytes exposed to ethanol (100 mmol/l), in the fatty livers of mice fed with ethanol (35), in the hearts of obese Zucker diabetic fatty *fa/fa* rats and *ob/ob* mice (36), as well as in cultured human skeletal muscle of obese type 2 diabetic patients (37). Inhibition of AMPK increases fatty acid synthesis by decreasing phosphorylation and increasing activity of ACC. In addition, increased production of malonyl-CoA decreases fatty acid oxidation by inhibiting carnitine palmitoyl transferase-1–mediated uptake of fatty acids into mitochondria (38). In addition, inhibition of AMPK increases the activity of sterol regulatory element–binding protein 1 and thereby increases expression of its target enzymes involved in fatty acid and triglyceride biosynthesis (5,26,35). Our data showing that exposure of HepG2 cells to high glucose inhibits the activity of AMPKα1, the major isoform in liver (14,31), but not AMPKα2 (which is likely due to the low baseline AMPKα2 activity), and decreases phosphorylation of ACC1 and ACC2, resulting in increased ACC activity and elevated hepatic lipids, suggests that inhibition of AMPKα1 activity in hepatocytes is responsible for the high-glucose–induced lipid accumulation.

ACC catalyzes the carboxylation of acetyl-CoA to form malonyl-CoA, an intermediate metabolite that plays a key role in the regulation of fatty acid metabolism (39). In mammals, there are two isoforms of ACC, ACC1 (265 kDa) and ACC2 (280 kDa). ACC1 is abundant in lipogenic tissues, such as liver and adipose tissues, where malonyl-CoA is the C_2 unit donor for de novo synthesis of long-chain fatty acids and for chain elongation of fatty acids to very-long-chain fatty acids. ACC2 is highly expressed in liver, skeletal muscle, and heart, where malonyl-CoA regulates fatty acid oxidation through inhibition of carnitine palmitoyltransferase I. Studies with ACC1 or ACC2 knockout mice demonstrate that because ACC1 and ACC2 are located in the cytosol or mitochondrial membrane, respectively, malonyl-CoA also exists in two different pools: the cytosolic pool, which is used as the precursor of fatty acid synthesis, and the mitochondrial pool, which regulates fatty acid oxidation (39). Both isoforms of ACC are phosphorylated and inactivated by AMPK (29,30). In the current study, phosphorylation of both ACC1 and ACC2 was significantly increased in HepG2 cells and in the liver of diabetic LDLR$^{-/-}$ mice by treatment with polyphenols, as indicated by immunoblots with phospho-ACC antibody. This is consistent with the observation that adiponectin increased phosphorylation of ACC1 and ACC2 in cultured primary hepatocytes and in mouse liver (31). In addition, activation of AMPK by metformin or leptin in vivo (5,40) and/or by AICAR in vitro (5) downregulates lipid synthesis and increases fatty acid oxidation. For instance, overexpression of constitutively active AMPK in the liver or treatment with metformin inhibits ACC activity or decreases expression of sterol regulatory element–binding protein 1 and its target genes in mouse liver (5,41). In the current study, three structurally related polyphenols increased both ACC1 and ACC2 phosphorylation, thereby decreasing their activities, and lowered triglyceride concentrations in HepG2 cells. As demonstrated in cells overexpressing a dominant-negative AMPK mutant, the effects of one of the polyphenols, S17834, were shown to be mediated by the activation of AMPK. Like the effect of the known AMPK activators, the lipid-lowering effect of polyphenols may be attributable to activation of AMPK and inactivation of ACC1 and ACC2 and consequently their effects to downregulate fatty acid synthesis and upregulate fatty acid oxidation.

Our data show that polyphenols promote AMPKα1 and -α2 activity as well as prevent the ability of high glucose to inhibit AMPKα1 activity, consistent with their ability to increase phosphorylation of AMPK and ACC. The activation of AMPK by polyphenols occurred relatively rapidly and well before any potential change in ATP level was detected. This further attests to the role of upstream signaling elements in mediating the rapid activation of AMPK and the inhibition of ACC by the polyphenols. The tumor suppressor LKB1 is an upstream kinase that has been implicated in the regulation of AMPK in several cells (25,42). LKB1 directly phosphorylates AMPK (25,42), and its role in lipid metabolism is evidenced by the observation that long-chain acyl-CoA esters inhibit phosphorylation of AMPK by LKB1/STRAD/MO25 (43). In addition, loss of liver LKB1 function in vivo results in hyperglycemia, loss of AMPK activity, and increased lipogenic gene expression in the liver, and it blocks the therapeutic effects of metformin (26). Because LKB1 may thus have a role in mediating the suppression of AMPKα1 activity and the accumulation of lipids in HepG2 cells exposed to elevated glucose, an effect of polyphenols on LKB1 is also possible.

Polyphenols have long been postulated to lower lipids through multiple mechanisms that have been implicated in the beneficial effects of tea and red wine on diabetic

cardiovascular disease (7,8). Apigenin and resveratrol, two polyphenols that are structurally similar to S17834, stimulated AMPKα1 and -α2 activation and ACC phosphorylation. This novel finding was made most evident in HepG2 cells exposed to high glucose, in which the two compounds prevented the decrease in AMPKα1 activity and ACC phosphorylation. Furthermore, both compounds decreased lipid accumulation caused by exposure to high glucose to the same extent as S17834. Although polyphenols were not reported previously to activate AMPK, other lipid-lowering mechanisms that are known to be stimulated by these compounds could potentially involve AMPK. For instance, polyphenols have been shown to stimulate PPAR-regulated gene expression (44). Soy isoflavones exert antidiabetic and hypolipidemic effects through PPAR pathways in obese Zucker rats and murine RAW 264.7 cells (44). The PPARγ agonist troglitazone, which has beneficial effects on lipids in diabetic patients, was shown to increase AMPK phosphorylation and to lower lipid levels in heart (36). Thus, we cannot exclude a role of PPAR activation in the effect of the polyphenols. Resveratrol and other polyphenolic compounds have recently been identified to be pharmacological activators of sirtuin 1 (Sirt1) (45), a longevity factor and an NAD-dependent protein deacetylase linked to life span extension whose activity is associated with calorie restriction (46). Stimulation of Sirt1 by resveratrol was shown to be key in decreasing lipid accumulation in cultured 3T3-L1 adipocytes by a mechanism involving corepression of PPARγ (20). Thus, PPARγ and/or Sirt1 could be involved in the role of polyphenols in AMPK activation and its lipid-lowering actions.

The AMPK activation and lipid-lowering effects of S17834 in HepG2 cells paralleled observations in vivo where S17834 increased AMPK and ACC phosphorylation and decreased lipid content in the liver of diabetic LDLR$^{-/-}$ mice. As is to be expected, AMPK stimulation by S17834 did not affect the severe hyperglycemia in the STZ-induced type 1 diabetic LDLR$^{-/-}$ mice because the inability of their pancreas to synthesize insulin precluded any potential effects mediated by altered insulin resistance. Thus, both in vivo and in vitro inhibition of AMPK, activation of ACC, and hepatocellular lipid accumulation caused by sustained high glucose levels was effectively opposed by activating AMPK with S17834.

It is very likely that the effects of S17834 on serum lipids, and in turn the attenuated atherogenesis that correlated with hyperlipidemia in diabetic LDLR$^{-/-}$ mice, is attributable to the same mechanisms by which we have shown that S17834 regulates AMPK activity and lipids in HepG2 cells. This is made more likely in this study by the fact that we used LDLR$^{-/-}$ mice. Because the major lipoprotein disposition mechanism is eliminated in these mice (16), serum levels of lipids largely reflect hepatic lipid synthesis. Although other mechanisms may have contributed, our results therefore suggest that S17834 exhibits its beneficial effect on aortic atherosclerosis in diabetic LDLR$^{-/-}$ mice in large part through its lipid-lowering effect, which is associated with its ability to stimulate hepatic AMPK activation.

In previous studies, we showed that S17834 decreased endothelial cell NADPH oxidase activity, inhibited adhesion molecule expression, and prevented atherogenesis in apoE$^{-/-}$ mice without an effect on serum lipids. Likewise, in this study the antiatherogenic action of S17834 in nondiabetic LDLR$^{-/-}$ mice could be mediated in a lipid-independent manner. Thus, although S17834 lacks direct oxygen radical scavenging activity (12), and its direct effects on NADPH oxidase occurred at concentrations higher than those that stimulate AMPK, it is likely that its ability to inhibit NADPH oxidase and adhesion molecule expression may have contributed to its effects on atherosclerosis in diabetic LDLR$^{-/-}$ mice. Indeed, AICAR inhibits NADPH oxidase in human neutrophils (47), and, like S17834, AICAR inhibits endothelial cell adhesion molecule expression (48). Nevertheless, the dramatic activation of AMPK by S17834 both in vitro and in vivo under conditions of high glucose and the demonstration that AMPK is required for its lipid lowering effects in HepG2 cells make it likely that the effects of S17834 on serum lipids, which correlated with its antiatherosclerotic effect in diabetic mice, are also mediated by AMPK. Thus, activation of AMPK may help to explain some of the antihyperlipidemic effects of polyphenols and provide an avenue for ameliorating hyperlipidemia and accelerated atherosclerosis in diabetes.

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