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Curcumin activates AMPK and suppresses gluconeogenic gene expression in hepatoma cells

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ABSTRACT

Curcumin, the bioactive component of curry spice turmeric, and its related structures possess potent anti-oxidant and anti-inflammatory properties. Several lines of evidence suggest that curcumin may play a beneficial role in animal models of diabetes, both by lowering blood glucose levels and by ameliorating the long-term complications of diabetes. However, current understanding of the mechanism of curcumin action is rudimentary and is limited to its anti-oxidant and anti-inflammatory effects. In this study we examine potential anti-diabetic mechanisms of curcumin, curcumin C3 complex®, and tetrahydrocurcuminoids (THC). Curcuminoids did not exert a direct effect on receptor tyrosine kinase activity, 2-deoxy glucose uptake in L6-GLUT4myc cells, or intestinal glucose metabolism measured by DPP4/α-glucosidase inhibitory activity. We demonstrate that curcuminoids effectively suppressed dexamethasone-induced phosphoenol pyruvate carboxy kinase (PEPCK) and glucose6-phosphatase (G6Pase) in H4IIE rat hepatoma and Hep3B human hepatoma cells. Furthermore, curcuminoids increased the phosphorylation of AMP-activated protein kinase (AMPK) and its downstream target acetyl-CoA carboxylase (ACC) in H4IIE and Hep3B cells with 400 times (curcumin) to 100,000 times (THC) the potency of metformin. These results suggest that AMPK mediated suppression of hepatic gluconeogenesis may be a potential mechanism mediating glucose-lowering effects of curcuminoids.

Introduction

Diabetes, a group of metabolic chronic disease affecting over 23 million people in the United States and over 250 million people worldwide, is characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both [1,2]. Plant derivatives with purported anti-diabetic properties have been used in folk medicine, traditional healing systems, and as complementary and alternative therapy. Several botanical, herbal, and biological products claim to lower blood glucose or decrease complications of diabetes (reviewed in Yeh et al. [3] and Mentreddy [4]). Examples of herbs that may regulate glucose homeostasis include *Aloe barbadensis*, *Eugenia jambolana*, *Gymnema sylvestre*, *Ocimum tenuiflorum*, *Trigonella foenum-graecum*, *Allum sativum*, *Galega officinalis* and *Curcuma longa* L. [4–6].

C. longa L. has been widely used for centuries in indigenous medicine for the treatment of a variety of inflammatory conditions and other diseases. Its medicinal properties are attributed to the yellow pigmented fraction, which primarily contains curcumin (~80%) and two other curcuminoids, desmethoxycurcumin (~10–20%) and bisdesmethoxycurcumin (less than 5%). Curcumin has been shown to possess potent anti-oxidant, anti-inflammatory, anti-cancer, and cardioprotective activities [5]. The molecular effects of curcumin have been shown to be mediated by transcription factors including NF-κB, PPAR-γ, and STAT; enzymes such as COX-2, iNOS, MAPK; inflammatory cytokines such as TNF-α, IL-1, IL-6, MCP; and growth factors such as EGF, HGF [7]. In humans and rodents, the systemic bioavailability of curcumin is low due to its inadequate absorption and rapid metabolic O-conjugation to curcumin glucuronide and curcumin sulfate, and bioreduction to tetrahydrocurcumin, hexahydrocurcumin and hexahydrocurcuminol [8]. Data from Phase I trials indicate that serum concentrations of curcumin ranged from 0.5 ± 0.11 μM in patients receiving 4000 mg/day to 1.77 ± 1.87 μM in patients receiving 8000 mg/day [9]. Another data from Phase II trials showed that only about 22–41 ng/ml curcumin were detectable in plasma even when 8 g of curcumin/day was given orally [10]. Despite its low bioavailability, several lines of evidence suggest that curcumin and tetrahydrocurcumin, may play a beneficial role in diabetes by lowering blood glucose levels in STZ-induced type 1 diabetic rats [11–13], STZ-nicotinamide induced type 2 diabetic rats [14], KK-A^y type 2 diabetic mice [15], *ob/ob* mice [16], and *db/db* mice [17]. Further, curcumin has been shown to be protective against nephropathy, retinopathy, and islet damage, in STZ-induced diabetic rats [18–20]. However, the molecular mechanisms mediating the glucose-lowering activ-

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ity of curcumin are poorly understood. In this study we have examined the effects of curcumin, curcumin C3 complex®, and tetrahydrocurcuminoids (THC), on metabolic targets that modulate insulin action, at concentrations comparable to its bioavailability. We report that the anti-diabetic effects of curcuminoids could, at least in part, be mediated by regulation of AMPK-mediated gluconeogenic gene expression.

Materials and methods

Reagents and antibodies. Curcumin (>90% purity) was purchased from Cayman Chemical Company (Ann Arbor, MI, USA). Curcumin C3 complex® (ratio-defined mixture of curcumin, bisdemethoxycurcumin, and demethoxycurcumin) and tetrahydrocurcuminoids (THC: ratio-defined mixture of tetrahydrocurcumin, tetrahydrobisdemethoxycurcumin, and tetrahydrodemothoxycurcumin) were gifts from Sabinsa Corporation (Piscataway, NJ). Curcumin, C3 complex®, and THC were dissolved in DMSO. Recombinant human insulin was purchased from Roche Diagnostics (Indianapolis, IL); dexamethasone from Sigma–Aldrich (St. Louis, MO); acarbose from LKT Laboratories (St. Paul, MN); phospho-AMPKalpha (Thr172) antibody from Cell Signaling Technology (Beverly, MA), and phospho-ACC2 (Ser79) antibody from Upstate Biotechnology (Charlottesville, VA).

Cell culture. Hep3B human hepatoma cells and H4IIE rat hepatoma cells were purchased from American Type Culture Collection (Manassas, VA, USA). Hep3B cells were cultured in Improved Modified Eagle's Medium (IMEM) and H4IIE cells in α-MEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. L6 muscle cells stably expressing myc-tagged GLUT4 (L6-GLUT4myc cells) were cultured as described previously [21]. Cytotoxicity of curcuminoids was analyzed using Cellstain double staining kit (DOJINDO, Rockville, MD) and fluorescence was visualized under a Nikon Eclipse TS100F microscope. Cell viability was quantitated using NIS Elements Basic Research software, version 3.01 (Nikon Instruments Inc., Melville, NY).

Insulin receptor tyrosine kinase activity (IR-TKA). Partially purified rat liver IRs, as described previously [22], were pre-incubated with various curcuminoids in the assay buffer (25 mM HEPES, pH 7.4, 0.1% Triton X-100, 0.05% BSA) for 30 min prior to 100 nM insulin stimulation for 10 min. An exogenous substrate, poly (Glu80Tyr20) was added and phosphorylation was carried out as described [22].

Glucose uptake assay. Serum-starved, confluent L6-GLUT4myc myoblasts were pre-incubated with various curcuminoids for 30 min before insulin (100 nM) treatment was for 30 min. Glucose uptake was assayed using [^{3}H] 2-deoxy-D-glucose (2-DOG), as described [23].

α-Glucosidase inhibitory assay. Curcuminoids were incubated with 50 μl of yeast α-glucosidase enzyme solution (Sigma–Aldrich) in 0.1 M phosphate buffer, pH 7.0 for 5 min. This was followed by the addition of 50 μl of *p*-nitrophenyl-α-D-glucopyranoside (Sigma) substrate solution and incubated for another 5 min at room temperature. Enzymatic inhibitory activity was quantitated by measuring the released p-nitrophenol at 405 nm.

Dipeptidyl peptidase inhibitory assay. Curcuminoids were incubated with porcine kidney dipeptidyl peptidase enzyme solution (Sigma–Aldrich) in 0.1 M Tris–HCl buffer, pH at 37 °C for 15 min, followed by the addition of 100 nmol of Gly-Pro-*p*-nitroanilde as substrate (Sigma–Aldrich). After incubation for another 15 min at room temperature, the amount of p-nitroaniline (pNA) released from Gly-Pro-pNA was measured at 405 nm using a microplate reader and calculated from a reference curve generated from Glu-Pro-p-nitroanilde standards.

Quantitative real-time PCR. RNA was extracted by using RNeasy Mini RNA isolation kit (QIAGEN, Valencia, CA). cDNA was synthesized by using iScript cDNA synthesis kit (BIO-RAD, Hercules, CA) and real-time PCR was performed using iQ SYBR Green Supermix (BIO-RAD) according to protocols recommended by the manufacturers. Primer sequences for Real-Time PCR were as follows: fructose 1,6 bisphosphatase (Fru1,6bPase) forward 5′-TCA TCG CAC TCT GGT CTA CG-3′, Fru1,6bPase reverse 5′-GCC CTC TGG TGA ATG TCT GT-3′; phosphoenol pyruvate carboxy kinase (PEPCK) forward 5′-GGG TGC TAG ACT GGA TCT GC-3′, PEPCK reverse 5′-GAG GGA GAA CAG CTG AGT GG-3′; glucose-6 phosphatase (G6Pase) forward 5′-GGG TGT AGA CCT CCT GTG GA-3′, G6Pase reverse 5′-GAG CCA CTT GCT GAG TTT CC-3′; β-Actin forward 5′-CCT CTA TGC CAA CAC AGT GC-3′, β-actin reverse 5′-CAT CGT ACT CCT GCT TGC TG-3′. Reaction conditions were as follows: 95 °C, 3:0 min; 95 °C, 0:15 min, 60 °C, 0:30 min, 72 °C, 0:30 min, repeated 40×; 55 °C, 0:10 min, repeated 80×. Expression levels were normalized to β-actin and gene expression was calculated as $2^{-\Delta\Delta C_T}$ and expressed as fold change, as described by Livak and Schmittgen [24]. All assays were carried out in triplicate.

Western blot analyses of phospho-AMPK and phospho-ACC. Confluent 6-well dishes of Hep3B or H4IIE cells were treated with curcuminoids, AICAR (0.5 mM), or metformin (2 mM) in serum-free media for 30 min. Cells were washed with ice-cold PBS three times and lysed in 300 μl of cell lysis buffer [50 mM HEPES, pH7.4, 100 mM sodium pyrophosphate, 100 mM EDTA, 20 mM sodium orthovanadate, 2 mM phenylmethylsulfonyl fluoride, 0.01 mg/ml aprotinin, 0.01 mg/ml leupeptin, 1% Triton X-100, and protease inhibitor cocktail tablet (Roche Diagnostics, Indianapolis)]. Cell lysate proteins were separated by 4–20% SDS–PAGE precast gels (Nu-Sep Inc., Austell, GA) followed by Western blot analysis using antibodies against phospho-AMPKα (Thr172) or phospho-ACC2 (Ser79). The membranes were stripped and reblotted for total AMPK and total ACC2 for loading equivalence. Chemiluminescence detection and image analysis were performed with UVP-Biochimie Bioimager and LabWorks software (UVP, Upland, CA).

Results and discussion

Curcuminoids do not improve IR activation or glucose uptake

First, we tested the effect of curcuminoids on both proximal and distal targets of insulin action, via IR activation and glucose uptake in skeletal muscle cells. Curcuminoids (curcumin, C3 complex®, and THC) did not improve *in vitro* IR-TK activity either in the presence or absence of insulin (Fig. 1A). Similarly, curcumin and THC had no effect on basal or insulin-stimulated glucose uptake in L6-GLUT4myc cells (Fig. 1B). Since C3 complex® demonstrated a tendency to lower insulin-stimulated IR-TK activity and higher concentrations (10 and 50 μM) of C3 complex® showed a significant inhibition of both basal and insulin-stimulated glucose uptake, these experiments were repeated with a wider range of concentrations of C3 complex® and similar results were observed (Supplemental data). The inhibition of glucose uptake by C3 complex® was not due to cytotoxic effects (Supplemental data). Our findings are consistent with earlier findings of Yang et al. [25], who showed that curcumin does not alter IR autophosphorylation, and Ikonomov et al. [26], who demonstrated that curcumin (100 μM) is an inhibitor of insulin-stimulated GLUT4 translocation and glucose transport in 3T3-L1 adipocytes. These findings indicate that curcumin's hypoglycemic effects are not mediated by an insulinomimetic activity, either at the level of the insulin receptor or through an upregulation of glucose transport.

Curcuminoids do not inhibit α-glucosidase activity or dipeptidyl protease-4 activity

Next, we analyzed the effect of curcuminoids on α-glucosidase, a membrane-bound intestinal enzyme that hydrolyzes oligosaccharides to glucose and other monosaccharides; and dipeptidyl

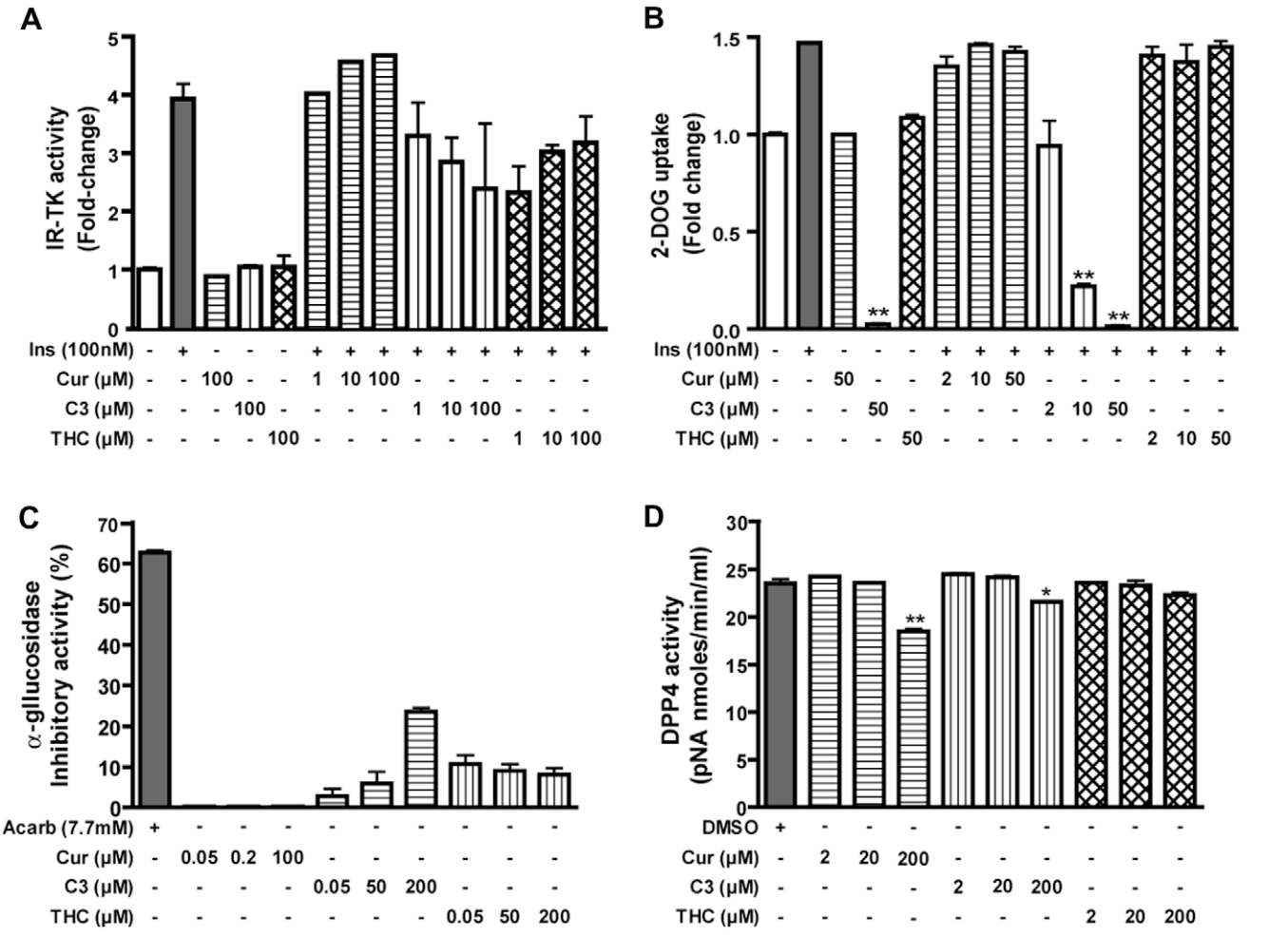

Fig. 1. Curcuminoids do not alter insulin receptor activation, glucose uptake into skeletal muscle cells, or α-glucosidase and dipeptidyl peptidase-4 (DPP4) enzyme activities. The effect of curcumin (Cur), curcumin C3 complex® (C3), tetrahydrocurcuminoids (THC) were assayed on insulin receptor tyrosine kinase (IR-TK) activity (A), glucose uptake in L6-GLUT4myc rat skeletal muscle cells (B), yeast α-glucosidase and DPP4 (D) enzymatic activities. Insulin (Ins) and acarbose (Acarb) were used as positive controls. Data are expressed as mean ± SEM. $^{*}p < 0.05$; $^{**}p < 0.01$, compared to basal.

peptidase (DPP4), a novel anti-diabetic drug target. Incretins such as glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide are released from the gut in response to food and which increase insulin secretion and play an essential role in maintaining glucose homeostasis. However, GLP-1 is rapidly cleaved and inactivated by DPP4. We demonstrate that acarbose, a commercial anti-diabetic drug, and used as positive control, inhibited α-glucosidase activity by 60% (Fig. 1C). Curcumin did not inhibit α-glucosidase at the concentrations tested, while curcumin C3 complex®, and THC demonstrated modest inhibition of α-glucosidase at higher concentrations (200 μM). Similarly, curcuminoids failed to inhibit DPP4 activity at lower concentrations (Fig. 1D), suggesting that these may not contribute to the mechanisms by which curcumin lowers blood glucose levels.

Curcuminoids suppress dexamethasone-induced gluconeogenic gene expression

Since impaired insulin-mediated suppression of gluconeogenesis and unregulated hepatic glucose production contributes to the fasting hyperglycemia observed in patients with diabetes [27], we studied the effects of curcumin on gluconeogenic gene expression in H4IIE rat hepatoma cells. Dexamethasone increased the gene expression of PEPCK and G6Pase by 3.5-fold, but not Fru1,6bPase due to the lack of glucocorticoid response element on the promoter region of Fru1,6bPase, as reported earlier report [28] (Fig. 2). Curcumin, curcumin C3 complex®, and THC decreased gene expression dose-dependently, primarily of G6Pase, at concentrations comparable to its bioavailability in H4IIE rat (Fig. 2A–C). Inhibition of PEPCK required higher concentrations (40–50 μM), but this was not due to cytotoxic effects (~75% of cells were viable at 50 μM curcumin treatment) (Fig. 2D). Further, similar to insulin, curcumin was also effective in inhibiting G6Pase expression in Hep3B human hepatoma cells (Fig. 3E).

Curcuminoids phosphorylate AMPK and ACC2

The AMP protein kinase (AMPK) is a cellular energy sensor inhibiting ATP-consumption and stimulating ATP-production under energy depleted condition [29]. Activation of AMPK is known to suppress gene expression of G6Pase and PEPCK and inhibit hepatic glucose production [30,31]. Further, activation of AMPK has been shown to phosphorylate and inactivate ACC, resulting in the inhibition of conversion of acetyl CoA to malonyl CoA for fatty acid synthesis, and increasing fatty acid oxidation [32]. We tested the effect of curcuminoids on AMPK activation in H4IIE and Hep3B hepatoma cells. As expected, both AICAR and metformin increased the phosphorylation of AMPK and ACC. Interestingly, curcuminoids induced an increase in phospho-AMPK and phospho-ACC levels equivalent to the activation by AICAR and metformin, but at 400

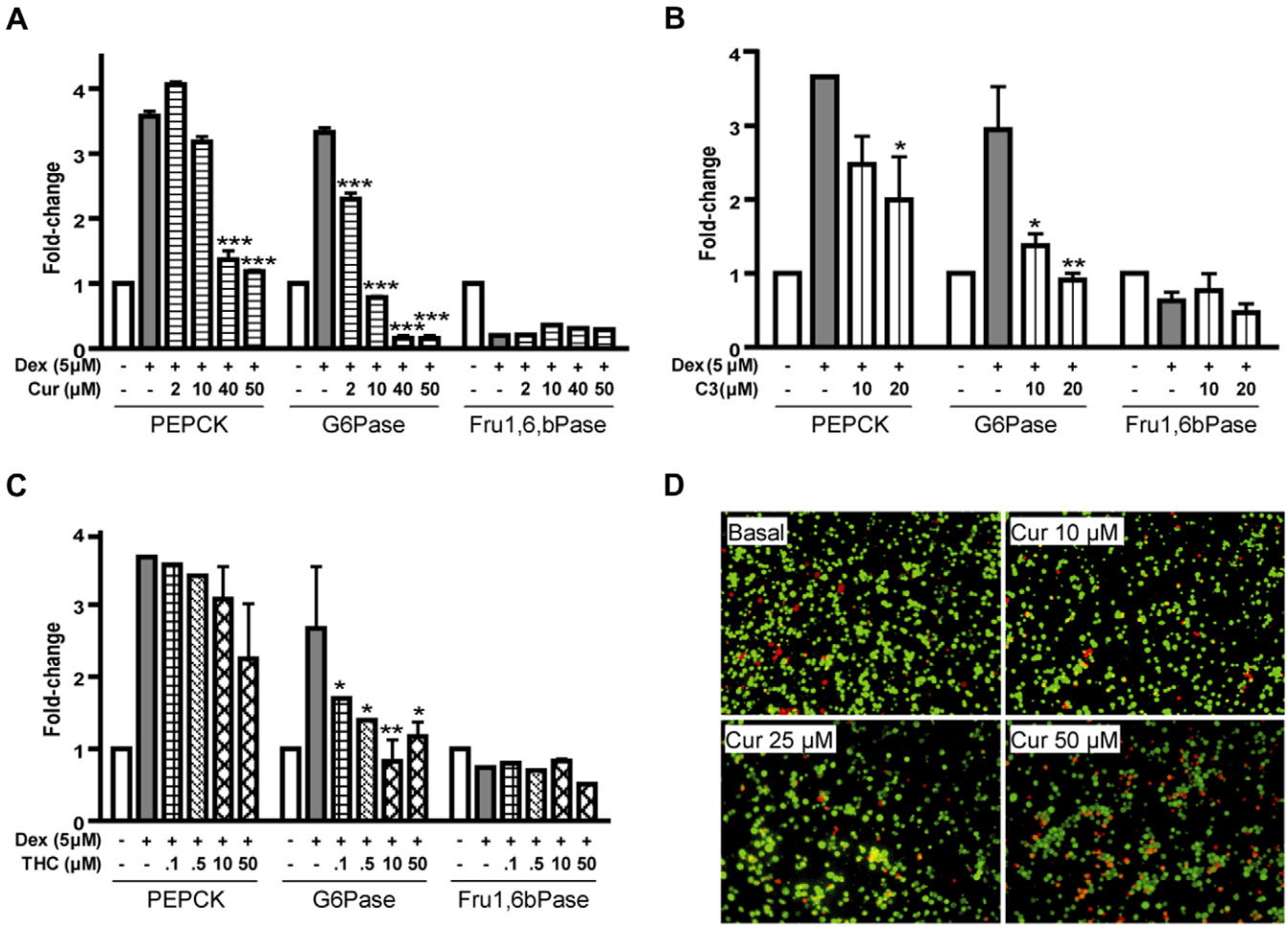

Fig. 2. Curcuminoids suppress dexamethasone-induced PEPCK and G6Pase expression in rat hepatoma cells. H4IIE rat hepatoma cells were treated with dexamethasone (Dex) and curcumin (A), C3 (B), THC (C) for 24 h. Total RNA was isolated and gene expression of PEPCK, G6Pase, and Fru1,6bPase were analyzed by real-time PCR. Data are expressed as fold change over basal relative to the housekeeping gene, β-actin (mean ± SEM). Cytotoxicity was assayed using calcein (green, viable cells) and propidium iodide (red, dead cells) fluorescence double staining (D) (20× magnification). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. (For interpretation of color mentioned in this figure legend the reader is referred to the web version of the article.)

times (curcumin) to 100,000 times (THC) the potency of metformin in H4IIE (Fig. 3A–C) and Hep3B cells (Fig. 3F). These doses were not cytotoxic (~75% viability at 50 μM curcumin treatment) (Fig. 3D). It is known that AMPK activation acutely stimulates glucose uptake by increasing GLUT4 gene expression in muscle cells [29], but our data shows curcuminoids do not have direct effect on glucose uptake in L6-GLUT4myc rat myoblasts or modulation of proximal insulin signal transduction.

Our studies demonstrate that THC was more effective in activating AMPK and inhibiting G6Pase gene expression compared to curcumin and C3 complex®. This is consistent with previous reports, which showed that THC was a better anti-diabetic and anti-oxidant agent than curcumin itself in rats, and with a better rate of absorption and solubility [15,33].

Our findings are consistent with those of Pari and Murugan [13] who showed that THC and curcumin restored the altered gluconeogenic enzymes to near normal levels in streptozotocin-induced diabetic rats. Additionally, Fujiwara et al. recently reported that curcumin inhibited glucose production in isolated mice hepatocytes by an insulin-independent manner by activation of AMPK and inhibition of G6Pase and PEPCK enzymatic activities [34]. Several polyphenols have been shown to activate AMPK, albeit by different mechanisms. Epigallocatechin-3-gallate (EGCG) activates AMPK mediated by Ca^{2+}/calmodulin-dependent protein kinase kinase (CaMKK) in rat primary hepatocytes [35], whereas resveratrol activates AMPK through SIRT1/LKB1, but not CaMKK in HepG2 hepatoma cells [36]. Additional studies are needed to identify the upstream kinase in curcumin-mediated activation of AMPK.

Animal experiments from various groups have shown that 0.22–1.5 μg/ml of curcumin remained in the animal blood after oral administration 1–2 g per kg to rats, whereas following i.p. administration of curcumin (0.1 g/kg) in rats, about 2.25 μg/ml appeared in their plasma, along with its metabolites dehydroxycurcumin, tetrahydroxycurcumin, and hexahydroxycurcumin [37,38]. Recent anti-cancer phase I clinical trials have shown that 12 g per day of curcumin administration was tolerable in humans, but the bioavailability of curcumin in humans is much lower than in animals [38–40]. However, despite the poor bioavailability, our results show that nanomolar concentrations of THC and low concentrations of curcumin (2–10 μM) can activate AMPK and suppress G6Pase activity.

In conclusion, these studies show that curcumin, and more effectively, its metabolite THC, activate AMPK and suppress hepatic gluconeogenic gene expression, which may explain some of the glucose-lowering effects of curcumin and THC. This novel mechanism of action of curcumin, along with its well-characterized anti-oxidant and anti-inflammatory properties, its tolerability, and promising findings from pre-clinical animal models of diabe-

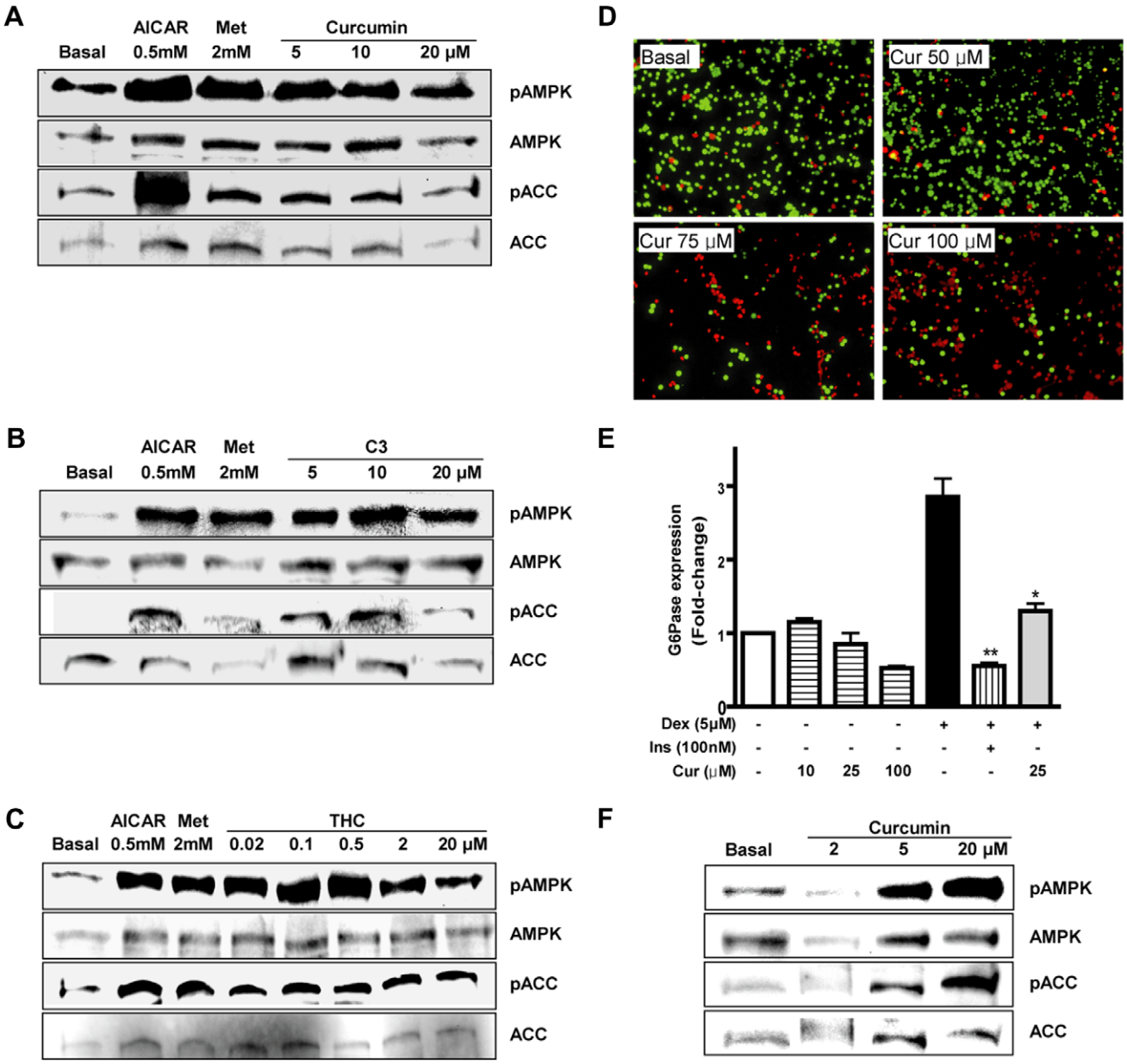

Fig. 3. Curcuminoids phosphorylate AMPK (AMP-activated protein kinase) and ACC2 (acetyl-CoA carboxylase 2) in hepatoma cells. H4IIE rat hepatoma cells (A–C) or Hep3B human hepatoma cells (D–F) were incubated with curcumin (A,F), C3 (B), or THC (C) for 30 min, and cell lysates were subjected to Western blot for pAMPKα (Thr173), total AMPK, pACC (Ser79), and total ACC. AICAR and metformin (Met) were used as positive controls; Cytotoxicity of curcumin-treated Hep3B human hepatoma cells was assayed using calcein (green, viable cells) and propidium iodide (red, dead cells) fluorescence double staining (D) (20× magnification); Curcumin's (Cur) effect on G6Pase gene expression in dexamethasone-treated (Dex) Hep3B human hepatoma cells (F). Insulin (Ins) was used as positive control. Data are expressed as fold change over basal, mean ± SEM (F). $^{*}p < 0.05$, $^{**}p < 0.01$. (For interpretation of color mentioned in this figure legend the reader is referred to the web version of the article.)

tes, suggest that curcuminoids may offer a complementary approach in the management of diabetes.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.08.018.

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