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Structural constraints and the importance of lipophilicity for the mitochondrial uncoupling activity of naturally occurring caffeic acid esters with potential for the treatment of insulin resistance

Hoda M. Eid [a], Diane Vallerand [a], Asim Muhammad [b], Tony Durst [b], Pierre S. Haddad [a], Louis C. Martineau [a,*]

[a] Natural Health Products and Metabolic Diseases Laboratory, Department of Pharmacology and Montreal Diabetes Research Center, Université de Montréal, Montreal, Quebec, Canada

[b] Department of Biology and Center for Research in Biopharmaceuticals and Biotechnology, University of Ottawa, Ottawa, Ontario, K1N 6N5 Canada

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ABSTRACT

Caffeic acid phenethyl ester (CAPE) has recently been shown to potently stimulate glucose uptake in cultured skeletal muscle cells through the AMPK pathway and therefore to have anti-diabetic potential. We report here that CAPE increases glucose uptake in C2C12 muscle cells by 225 ± 21% at 50 μM, and that activation of AMPK is a consequence of the metabolic stress resulting from an uncoupling-type disruption of mitochondrial function (complete uncoupling at 50 μM). We also observe that the therapeutic potential of CAPE is offset by its high potential for toxicity. The purpose of this study was therefore to identify other active caffeic acid derivatives, evaluate their ratio of activity to toxicity, and elucidate their structure–activity relationship. Twenty naturally occurring derivatives were tested for glucose-uptake stimulating activity in C2C12 cells following 18 h of treatment and for uncoupling activity in isolated rat liver mitochondria. Cytotoxicity was assessed in C2C12 cells by the release of lactate dehydrogenase over 18 h. In addition to CAPE, four compounds were identified to be active, both stimulating glucose uptake and uncoupling isolated mitochondria. Activity required that the caffeic acid moiety be intact and that the compound not contain a strongly ionized group. Both activity and toxicity were found to be well-correlated to predicted lipophilicity. However, two compounds exhibited little to no toxicity while still stimulating glucose uptake by 65–72%. These results support a therapeutic potential for this family of compounds and provide the framework for the design of alternatives to Metformin with an optimized balance of safety and activity.

1. Introduction

Insulin resistance is a major health concern throughout the world. It is a precursor to the diseases that make up the metabolic syndrome, including type II diabetes, cardiovascular disease, and nonalcoholic fatty liver disease, and is linked to excess adiposity, sedentary lifestyle, poor dietary habits, and aging [1]. At the cellular level, the development of insulin resistance is caused in part by abnormal accumulation and metabolism of lipids, as well as by mitochondrial dysfunction [2,3].

A key therapeutic target of pharmacological interventions for improving insulin sensitivity is the master metabolic regulatory enzyme AMP-activated protein kinase (AMPK) [4,5]. This enzyme is an extremely sensitive monitor of energy homeostasis, specifically of the concentrations of ATP and AMP. Upon activation under conditions of metabolic stress, AMPK triggers cytoprotective programs for acutely upregulating ATP production and down-regulating non-essential energy expenditure, as well as transcriptional events that confer enhanced protection against future metabolic stress [6,7]. In the context of insulin resistance and compromised glycemic control, the activation of AMPK produces insulin-like effects that contribute to the normalization of hyperglycemia, namely the inhibition of glucose output by liver cells and the stimulation of glucose uptake by skeletal muscle cells. Furthermore, acute stimulation of fat oxidation and increased mitochondrial density are also AMPK-mediated effects that are relevant to the protection and restoration of insulin sensitivity in liver and muscle.

AMPK mediates the actions of the successful insulin-sensitizer Metformin and of the other members of the biguanide family [8]. These compounds indirectly activate AMPK by inducing a partial and transient inhibition of mitochondrial energy transduction and

* Corresponding author at: Department of Pharmacology, Université de Montréal, P.O. Box 6128 Station Centre-Ville, Montréal, Québec, H3C 3J7 Canada. Tel.: +1 514 343 6111; fax: +1 514 343 2291.
E-mail address: l.martineau@umontreal.ca (L.C. Martineau).

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thereby disrupting energy homeostasis. The biguanides are effective at inhibiting hepatic glucose production [9,10]. However, their effect on skeletal muscle, the major site of glucose disposal, is more limited [11]. Also, Metformin and the other biguanides are associated with a potential for toxicity in the form of lactic acidosis [12] since compromised aerobic metabolism must be compensated by upregulation of anaerobic glycolysis. It appears that this potential is proportional to activity and that safety can only be improved at the cost of efficacy. Indeed, the more powerful biguanides have been removed from many markets, leaving only Metformin [9,10], a product requiring doses of multiple grams per day.

There is an impetus to identify novel activators of AMPK that are at least as safe as Metformin but that are more potent and more efficacious at stimulating glucose uptake and inducing other therapeutically relevant AMPK-mediated effects in skeletal muscle. A starting point may be other types of compounds that also disrupt mitochondrial function but which do so through different mechanisms. Inhibitors of ATP synthase [13–15] or dissipators of the mitochondrial proton gradient that drives conversion of ADP to ATP (i.e. uncouplers) are found in nature where they are used by many plant species to defend against predatory microorganisms [13,16]. While lactic acidosis will always remain a potential complication of any disruptor of aerobic metabolism, it may nevertheless be possible to identify in nature classes of compounds with a more favorable toxicity-to-activity relationship, especially if focus is placed on small phenolic compounds presumably easily metabolized by higher organisms. Indeed, activity may be uncoupled from this type of toxicity if mitochondrial effects are short-lived and of no more than sufficient duration to promote the activation of AMPK. Of interest is the naturally occurring small phenolic caffeic acid phenethyl ester (CAPE). This compound has recently been observed to activate AMPK kinase and to robustly and potently stimulate glucose uptake in skeletal muscle cells [17]. The purpose of the present study was to assess whether this effect of CAPE is a consequence of disruption of mitochondrial function and to test related compounds for similar activity. The results indicate that CAPE is an uncoupler of oxidative phosphorylation, that other closely related derivatives also exhibit both uncoupling activity and glucose-uptake stimulating activity, and that some of these compounds such as caffeic acid ethyl ester (CAEE) and caffeic acid methyl ester (CAME) exhibit useful activity with little to no associated cytotoxicity.

2. Materials and methods

2.1. Source of compounds and reagents

CAPE and other caffeic acid derivatives (summarized in Table 1 and Fig. 4) were purchased from Sigma–Aldrich (Oakville, ON), with the exception of caffeic acid methyl ester, dihydrocaffeic acid, rosmarinic acid, ferulic acid methyl ester and ferulic acid ethyl ester purchased from Indofine Chemical Co. (Hillsborough, NJ), caffeic acid n-octyl ester and ferulic acid phenethyl ester purchased from LKT Laboratories Inc. (St.-Paul, MN), and dihydrocaffeic methyl ester, 4-hydroxycinnamic methyl ester, ferulic acid methyl ester, 2,4-dihydroxycinnamic acid methyl ester, and 4-(1-propenyl)-catechol synthesized as described in Sections 2.2 and 2.3. Cell culture reagents were purchased from Invitrogen Life Technologies (Burlington, ON), unless otherwise noted. Other reagents were purchased from Sigma–Aldrich unless otherwise noted. Antibodies against phosphorylated (Ser 79) and pan-specific acetyl-CoA-carboxylase (ACC), phosphorylated (Ser 473) and pan-specific Akt, and β-actin were purchased from Cell Signaling Technology (Danvers, MA). Secondary HRP-conjugated antibodies were purchased from Jackson Immunoresearch (Cedarlane Laboratories, Hornby, ON).

2.2. Synthesis of methyl ester compounds

Solutions of dihydrocaffeic acid, 4-hydroxycinnamic acid, ferulic acid, and 2,4-dihydroxycinnamic acid (11.1 mM) in methanol (50 ml) were separately treated with a catalytic amount of concentrated H_2SO_4 and heated at reflux for 10 h. The reaction mixtures were cooled at room temperature and concentrated. The residues were dissolved in ethyl acetate and washed successively with water and brine. The ethyl acetate layers were dried over anhydrous $MgSO_4$ and purified by column chromatography on silica gel to give dihydrocaffeic methyl ester (yellowish brown liquid), 4-hydroxycinnamic methyl ester (white powder), ferulic acid methyl ester (yellowish brown liquid), and 2,4-dihydroxycinnamic acid methyl ester (white powder). The identity and purity of these four compounds were confirmed by mass spectroscopy and by ^1H and ^{13}C NMR spectroscopy (Avance 400; Bruker BioSpin Corp., Billerica, MA).

2.3. Synthesis of 4-(1-propenyl)-catechol

4-(1-Propenyl)-catechol was synthesized according to a previously reported method [18]. Briefly, 3,4-dihydroxybenzaldehyde (7.24 mmol) and imidazole (2.3 equivalents) were dissolved in a 1:1 DMF/THF solution (10 ml). Teriary butyl dimethylsilyl chloride (2.2 equivalents) and 4-dimethylaminopyridine (trace) were added and the reaction mixture was stirred overnight at room temperature. The mixture was then diluted with distilled water (15 ml) and ether (25 ml) and then extracted with ethyl acetate (3 ml × 15 ml). The organic extracts were combined, dried over $MgSO_4$, filtered, and evaporated under vacuum. The crude product was purified on a flash column. Elution with hexanes resulted in 1-(3,4-bis(tert-butyldimethylsilyloxy) benzaldehyde (compound 1) as a clear colorless oil. Ethylmagnesium bromide (3.0 M solution in THF; 1.5 equivalents) was added dropwise to a solution of compound 1 (1.09 mmol) in 10 ml of dry THF under nitrogen atmosphere at 0 °C. The resulting grayish solution was stirred for 30 min at 0 °C and left at room temperature for 1 h. The organic mixture was diluted with 10% HCl solution (2 ml) and extracted with ethylacetate (3 ml × 10 ml). The organic layers were combined, dried over $MgSO_4$, filtered and concentrated under vacuum. The crude product was purified on a silica column. Elution with 15% ethylacetate in hexanes resulted in compound 2 as yellow oil. Compound 2 (0.75 mmol) was dissolved in methanol (5 ml) and few drops of concentrated HCl were added. The reaction mixture was refluxed for 2 h. The resulting yellowish mixture was diluted with distilled water and extracted with ethylacetate (3 ml × 10 ml). The organic layers were combined, dried over $MgSO_4$, filtered and concentrated under vacuum. The crude product was purified on a silica gel column. Elution with 50% ethylacetate in hexanes resulted in 4-(1-propenyl)-catechol as yellow solid at a purity of greater than 95%. The identity and purity of this compound was confirmed by mass spectroscopy and by ^1H and ^{13}C NMR spectroscopy.

2.4. Estimation of pK_a and log P

The acid-dissociation constant (pK_a) for each ionizable group and the octanol–water partition coefficient (P), a predictor of lipophilicity, were estimated using the Marvin 5.1 Academic Package (ChemAxon Kft., Budapest, Hungary). Structures were inputted manually into MarvinSketch. The Marvin Protonation calculator plug-in was used to calculate pK_a at a temperature of 37 °C. The Marvin Partitioning calculator plug-in was used to

Table 1
Physicochemical parameters and measured activities of test compounds.

Compound	Predicted pK_a	Predicted log P	Isolated mitochondria		Skeletal muscle cells	
			Uncoupling effect (50 μM)	Residual capacity (50 μM)	ΔGlucose uptake (50 μM)	ΔViability (50 μM)
Caffeic acid	3.1; 9.3; 12.7	1.5	−1% ± 1%	90% ± 4%	+4% ± 9%	
Caffeic acid, dihydro ethyl ester	9.3; 12.7	2.0	0% ± 0%	115% ± 13%	+12% ± 5%	
Caffeic acid, dihydro methyl ester	9.3; 12.7	1.6	0% ± 1%	104% ± 3%	+28% ± 5%	
Caffeic acid, dihydro phenethyl ester	9.3; 12.7	3.6	2% ± 1%	80% ± 14%	+13% ± 7%	
Caffeic acid, ethyl ester (CAEE)	9.2; 12.6	2.3	14% ± 1%	86% ± 10%	+72% ± 6%	−3% ± 1%
Caffeic acid, methyl ester (CAME)	9.2; 12.6	1.9	7% ± 4%	85% ± 9%	+65% ± 12%	0% ± 1%
Caffeic acid, n-octyl ester (CAOE)	9.2; 12.6	5.0	48% ± 1%	18% ± 3%	+230% ± 20%	−16% ± 6%
Caffeic acid, phenethyl ester (CAPE)	9.2; 12.6	3.9	105% ± 20%	0% ± 0%	+225% ± 21%	−14% ± 6%
Caffeic acid, 1,1-dimethylallyl ester (CAAE)	9.2; 12.6	3.3	92% ± 10%	3% ± 3%	+158% ± 15%	−9% ± 4%
Chlorogenic acid	3.3; 9.2; 12.5	−0.3	2% ± 1%	105% ± 3%	−6% ± 7%	
Cinnamic acid	4.0	2.1	1% ± 0%	93% ± 5%	+4% ± 2%	
Cinnamic acid, 2,4-dihydroxy methyl ester	8.7; 10.7	1.9	1% ± 0%	97% ± 1%	−7% ± 6%	
Cinnamic acid, 2,5-dihydroxy methyl ester	9.5; 11.3	1.9	−1% ± 1%	90% ± 13%	+4% ± 6%	
Cinnamic acid, methyl ester	n/a	2.5	0% ± 1%	87% ± 1%	+14% ± 6%	
Cinnamic acid, 4-hydroxy methyl ester	9.4	2.2	1% ± 0%	104% ± 8%	+2% ± 4%	
Ferulic acid	3.3	1.7	0% ± 0%	108% ± 8%	−6% ± 7%	
Ferulic acid, ethyl ester	9.9	2.4	1% ± 2%	104% ± 7%	−10% ± 5%	
Ferulic acid, methyl ester	9.9	2.1	0% ± 0%	93% ± 8%	+11% ± 13%	
Ferulic acid, phenethyl ester	9.9	4.1	0% ± 1%	87% ± 1%	−68% ± 2%	
4-(1-Propenyl)-catechol	9.3; 12.7	2.5	0% ± 1%	107% ± 5%	+11% ± 3%	
Rosmarinic acid	3.1	3.0	0% ± 1%	103% ± 3%	+6% ± 5%	

Notes: pK_a = acid dissociation constant; P = octanol–water partition coefficient for the neutral form of compound; n/a = not applicable. Data are expressed as mean ± SEM. Respiration data were collected from 2 separate experiments performed in duplicate. Calculations of uncoupling effect and of residual mitochondrial capacity are described under Section 2. Residual mitochondrial capacity is the net result of the uncoupling effect and any inhibition of respiration or of ATP synthase. Glucose uptake data are expressed relative to the vehicle control group (SEM = 8%) and were collected from 3 separate experiments performed in triplicate. Treatment duration was 18 h. Viability was calculated from % of total LDH released into the medium. Viability data are expressed relative to vehicle control group (SEM = 1%) and were collected from 2 separate experiments performed in triplicate. Treatment duration was 18 h.

calculate log P of the neutral molecular species at an ionic strength of 0.1 M/dm^3 Na^+/K^+ and 0.1 M/dm^3 Cl^-. Calculated log P and pK_a were verified against published experimental values whenever these were available.

2.5. Cell culture

C2C12 murine skeletal myoblasts were obtained from the American Type Culture Collection (ATCC; Manassas, VA). C2C12 myoblasts were cultured in 6- or 12-well plates at 37 °C in a 5% CO_2 environment in high-glucose Dulbecco's Modified Eagle's Medium (DMEM; Wisent, St-Bruno, QC) containing 10% fetal bovine serum and 10% horse serum (HS) and supplemented with antibiotics (penicillin 100 U/ml, streptomycin 100 μg/ml) as previously described [19–21]. Upon reaching 80% confluence, serum content was reduced to 2% to induce differentiation into multinucleated myotubes over a period of 7 days. On the 6th day of differentiation, compounds solubilized in dimethyl sulfoxide (DMSO) and mixed in culture medium to achieve a final concentration of 50 μM in 0.1% DMSO were applied for 18 h prior to glucose uptake, western immunoblot, or LDH release assays.

2.6. ^{3}H-deoxyglucose uptake assay

Differentiated C2C12 myotubes grown in 12-well plates were treated for 18 h with 0.1% DMSO (vehicle control) or with 50 μM of CAPE or other caffeic acid derivative. The effects of CAPE resulting from a treatment of this duration have not previously been reported. The 50 μM concentration was selected for the testing of all compounds based on pilot studies indicating that 50 μM or below of a wide variety of naturally occurring small phenolics is typically well-tolerated by C2C12 myotubes over an 18 h period, with no effect on morphology and little to no effect on viability. Furthermore, a pilot dose–response study of CAPE indicated that glucose uptake following an 18 h treatment peaked at 50 μM (not shown). Following treatment, cells were rinsed twice with Krebs-phosphate buffer (KPB; 20 mM HEPES, 4.05 mM Na_2HPO_4, 0.95 mM NaH_2PO_4, pH 7.4, 136 mM NaCl, 4.7 mM KCl, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 5 mM glucose, 0.5% BSA) at 37 °C and equilibrated in this buffer for 30 min. Some vehicle-control cells were treated during this time with 100 nM insulin to serve as a reference control. Following this, cells were washed twice in glucose-free KPB at 37 °C and then incubated for exactly 10 min in 0.5 μCi/ml of 2-deoxy-D-[1-^{3}H]-glucose (TRK-383; Amersham Biosciences, Baie d'Urfé, QC) in this same buffer. Cells were then rapidly placed on ice and rinsed three times with ice-cold KPB, before lysis and scraping in 1 ml of 0.1 mM NaOH. Lysates were added to 4 ml of scintillation liquid cocktail (Ready-Gel 586601; Beckman Coulter Inc., Fullerton, CA) and radioactivity was measured in a scintillation counter (LKB Wallac RackBeta; Perkin Elmer, Montreal, QC).

2.7. Isolation of mitochondria from rat liver

Mitochondria were isolated from the liver of male Wistar rats (Charles River, St-Constant, QC) weighing between 225 and 250 g. Rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (50 mg/kg body weight) and underwent laparotomy. All experimental procedures were approved by the Université de Montréal Animal Experimentation Ethics Committee and animals were treated in accordance with guidelines of the Canadian Council on the Care and Protection of Animals. The portal vein was canulated while the hepatic artery and the infrahepatic inferior vena cava were ligated. The livers were flushed with 100 ml of Krebs–Henseleit buffer (25 mM $NaHCO_3$, 1.2 mM, KH_2PO_4, pH 7.4, 137 mM NaCl, 4.8 mM KCl, 2.1 mM $CaCl_2$, 1.2 mM $MgSO_4$) at ambient temperature and livers were removed and placed on ice. Mitochondria were isolated from 1 g of liver as described by Johnson and Lardy. Briefly, tissue was homogenized on ice using a Teflon potter homogenizer in ice-cold isolation buffer (10 mM Tris, pH 7.2, 250 mM sucrose, 1 mM EGTA). The homogenate was centrifuged at 600 × g for 10 min at 4 °C in order to remove cellular fragments. The supernatant was recovered and centrifuged at

12,000 × *g* for 6 min at 4 °C. The supernatant was discarded and the pellet was washed in ice-cold isolation buffer and recentrifuged. The pellet was then washed in EGTA-free isolation buffer, and again recentrifuged. The final pellet containing viable mitochondria was resuspended in ice-cold EGTA-free isolation buffer and this preparation was kept on ice until respiration experiments. Protein content of the preparation was determined according to the Lowry method.

2.8. Mitochondrial respiration assay

O_2 consumption was measured at 25 °C using a Clark-type oxygen microelectrode in a 1 ml volume temperature-controlled chamber with oxygen concentration sampled and recorded to a microcomputer at a frequency of 1 Hz (Oxygraph system; Hansatech Instruments, Norfolk, England) as previously described. Briefly, 1 mg of mitochondrial protein was added to 990 μl of respiration buffer (5 mM KH_2PO_4, pH 7.2, 250 mM ultra-pure sucrose, 5 mM $MgCl_2$, 1 mM EGTA). Mitochondrial respiration was initiated by addition of the complex II substrate succinate (5 mM final concentration). After reaching a stable rate of basal O_2 consumption (RBOC; State 4 respiration), vehicle alone or a caffeic acid derivative solubilized in DMSO was injected to achieve a final concentration of 50 μM of experimental compound in 0.1% DMSO. An increase in RBOC per mg mitochondrial protein was considered an uncoupling effect. DMSO used at 0.1% did not affect RBOC. Basal respiration was allowed to proceed for at least 30 additional seconds before the induction of oxidative phosphorylation (State 3 respiration) by the addition of 200 μM (final concentration) ADP. Each experimental session consisted of 10–12 experiments from a single mitochondrial preparation, including 3–4 vehicle control experiments to determine baseline values for the session. The effect of each experimental compound was evaluated as: (1) the increase in RBOC per mg protein (a measure of the magnitude of the uncoupling effect); (2) the decrease in functional capacity (FC) per mg protein (a measure of the magnitude of the uncoupling effect plus any additional inhibitory effect), where FC was defined as the difference of the rate of ADP-stimulated O_2 consumption (RASOC) per mg protein (maximal functional rate of consumption) and RBOC per mg protein (rate of consumption driven by proton leak and not contributing to ATP synthesis). Calculations were as follows: the average FC per mg protein of the vehicle control experiments for a given session was calculated by subtracting the average RBOC per mg protein from the average RASOC per mg protein. For (1) above, the absolute increase in RBOC per mg protein measured in a given experiment was expressed as a percentage of the average control FC per mg protein for the session. For (2) above, the FC per mg protein measured in a given experiment was expressed as a percentage of the average control FC per mg protein for the session to give the % residual FC. All compounds were tested in at least two different mitochondrial preparations.

2.9. Western immunoblot

Differentiated C2C12 myotubes grown in 6-well plates were treated for 18 h with 0.1% DMSO or with 50 μM of CAPE or of other caffeic acid derivative, and lysed for western immunoblot analysis. Some vehicle-control cells were treated with 2 mM 5-aminoimidazole-4-carboxamide-1-b-D-rubofuranozide (AICAR; Toronto Research Chemicals, North York, ON), a positive control for activation of the AMPK pathway, for 30 min immediately prior to lysis. Following treatment, plates were placed on ice and washed three times in ice-cold phosphate-buffered saline (PBS; 8.1 mM $NaHPO_4$, 1.5 mM KH_2PO_4, pH 7.4, 137 mM NaCl and 2.7 mM KCl) and lysed in 250 μl of lysis buffer (25 mM Tris–HCl pH 7.4, 25 mM NaCl, 0.5 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate 0.1% SDS) containing a commercial cocktail of protease inhibitors (Complete Mini; Roche, Mannheim, Germany) supplemented with 1 mM phenylmethanesulfonyl fluoride, as well as a cocktail of phosphatase inhibitors (1 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 10 mM sodium fluoride). Cells were allowed to lyse for 15 min on ice, and then scraped into microcentrifuge tubes, periodically vortexed, and centrifuged at 600 × *g* for 10 min at 4 °C. Supernatants were decanted and stored at −80 °C until further analysis. Protein content was assayed by the bicinchoninic acid method (Thermo Scientific Pierce Protein Research, Rockford, IL) standardized to bovine serum albumin. Lysates were diluted to a concentration of 1.25 mg total protein per ml and boiled for 5 min in reducing sample buffer (62.5 mM Tris–HCl pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol and 0.01% bromophenol blue). 20 μg of protein of each sample were separated on 10% polyacrylamide mini-gels and electrotransferred to polyvinylidene fluoride membrane (Millipore, Bedford, MA) overnight under 330 mA of current at 4 °C. Membranes were blocked for 2 h at room temperature with 5% skim milk in Tris-buffered saline (20 mM Tris–HCl, pH 7.6 and 137 mM NaCl) containing 0.1% Tween 20 (TBST). Membranes were then incubated overnight at 4 °C in blocking buffer with primary antibodies at a concentration of 1:1000. Membranes were washed 5 times with TBST and incubated 1.5 h at ambient temperature in TBST with appropriate horseradish peroxidase-conjugated secondary antibodies at 1:50,000–100,000. Revelation was performed using the enhanced chemiluminescence method (Amersham Biosciences, Buckinghanshire, England) and blue-light-sensitive film (Amersham Biosciences). Experiments were repeated on 3 different passages of cells, each passage containing all conditions in parallel. All samples from a given passage were separated and transferred simultaneously to a single membrane. Quantification of the integrated density of bands was performed using a flatbed scanner (ScanJet 6100; Hewlett Packard, Palo Alto, CA) and NIH Image 1.63 software (National Institutes of Health, Bethesda, MD).

2.10. LDH release cytotoxicity assay

Differentiated C2C12 myotubes grown in 12-well plates were treated for 18 h with 0.1% DMSO or with 50 μM of CAPE or of other caffeic acid derivative. Medium was removed and kept on ice. Cells were rinsed in PBS and lysed in 1% Triton X-100. Lactate dehydrogenase (LDH) activity in medium and in lysates was assayed with the LDH-Cytotoxicity Assay Kit II (BioVision, Mountain View, CA). Medium LDH activity was expressed as a percentage of total (medium + lysate) LDH activity. Experiments were performed in triplicate.

2.11. Statistical analysis

All data are reported as the mean ± SEM of the indicated number of experiments. Results were analyzed by one-way analysis of variance using StatView software (SAS Institute Inc., Cary, NC). Statistical significance was set at $p \leq 0.05$. Non-linear regression analysis was performed by Prism 4.0 (GraphPad Software Inc., La Jolla, CA) using the following sigmoidal dose–response equation: y = bottom + (top − bottom)/$(1 + 10^{((x50 - x)^{*}\ \text{Hill slope})})$ where $x = \log P$ and $x_{50} = \log P$ resulting in half maximal effect.

3. Results

3.1. CAPE increases glucose uptake following an 18 h treatment

CAPE has recently been shown to induce an important AMPK-mediated stimulation of glucose uptake in skeletal muscle cells

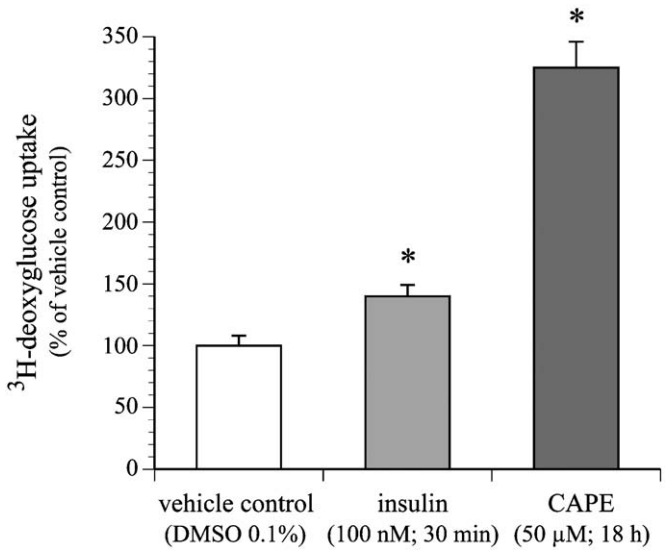

Fig. 1. Caffeic acid phenethyl ester (CAPE) increased non-insulin-stimulated (basal) ^{3}H-deoxyglucose uptake in differentiated C2C12 skeletal muscle cells by more than 3-fold. Cells were treated with 50 μM of CAPE or with vehicle (0.1% DMSO) for 18 h. The effect of CAPE was 5.7-fold greater than that of 100 nM insulin applied acutely for the last 30 min of the treatment in vehicle-treated cells. Data are expressed normalized to basal uptake of the vehicle control group. Data are presented as the mean of 3 experiments ± SEM, each experiment composed of 3–4 replicates per condition. * Indicates a significant ($p \leq 0.05$) difference from the vehicle control group, as assessed by ANOVA.

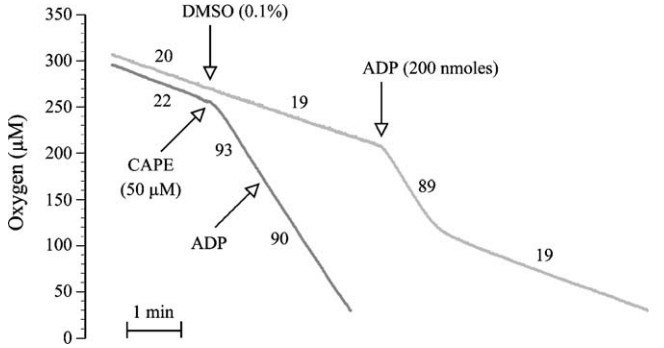

Fig. 2. CAPE induced a powerful uncoupling effect in isolated rat liver mitochondria. Representative tracings of succinate-supported basal (State 4) and ADP-stimulated (State 3) O_2 consumption, assessed at 25 °C with a Clark-type oxygen electrode. The uncoupling effect of CAPE at 50 μM (dark tracing) was complete in that the rate of basal O_2 consumption was increased to slightly more than the rate of ADP-stimulated O_2 consumption in vehicle-treated mitochondria (light tracing) and the addition of ADP did not further increase O_2 consumption. Values represent rate of consumption in nmoles O_2 per mg mitochondria per minute.

following a 1 h treatment [17]. A first objective of the present study consisted in testing the effect of CAPE on muscle cell glucose uptake following a longer treatment duration more conducive to the expression of AMPK-mediated transcriptional effects. Our previous studies on natural products that enhance basal glucose uptake through AMPK have shown that in C2C12 muscle cells an 18 h treatment invariably results in a more important effect than a 1 h treatment [19–21], presumably due to transcriptional effects of AMPK [6,7,22,23]. Differentiated C2C12 cells were therefore treated with CAPE (50 μM) or with vehicle (0.1% DMSO) alone for 18 h prior to performing a ^{3}H-deoxyglucose uptake assay in the absence of insulin. CAPE was found to increase basal rate of uptake by 225% (Fig. 1). This effect was 5.7-fold greater than the effect of 100 nM of insulin applied to vehicle-control cells 30 min prior to the uptake assay. The effect of CAPE was also superior to that of Metformin, which typically only induces a 25–40% increase in uptake following an 18 h treatment in C2C12 muscle cells [19–21,24].

3.2. CAPE is an uncoupler

The effects of CAPE on the respiration of isolated rat liver mitochondria were assessed in order to test the hypothesis that the reported activation of AMPK by CAPE [17] and the remarkable increase in basal glucose uptake observed above were the result of a metabolic stress induced by the disruption of energy transduction pathways. This hypothesis was appropriate in light of the known effects of several naturally occurring compounds on mitochondrial oxidative phosphorylation [13,16,25,26]. CAPE (50 μM) was observed to completely uncouple mitochondrial oxidative phosphorylation, whereby the rate of basal oxygen consumption in CAPE-treated mitochondria was increased approximately 4.5-fold, to the same rate as that achieved with ADP stimulation in vehicle-treated mitochondria, and the addition of ADP to CAPE-treated mitochondria did not further increase oxygen consumption (Fig. 2). This uncoupling effectively abolished ATP synthetic capacity.

3.3. CAPE decreases cellular viability

In light of CAPE's important effect on the function of isolated mitochondria, it was hypothesized that the treatment of cells with CAPE could be cytotoxic and compromise viability. Cellular viability was therefore assessed in C2C12 cells treated for 18 h with CAPE (50 μM) or with vehicle alone by assaying the release of the enzyme LDH as a function of total LDH content. In vehicle-treated cells, LDH release over this time was measured to be 7.6% of total LDH content, whereas in CAPE-treated cells, release was increased to 20.7% (Fig. 3). This corresponded to a decrease in viability of 14%, relative to vehicle control.

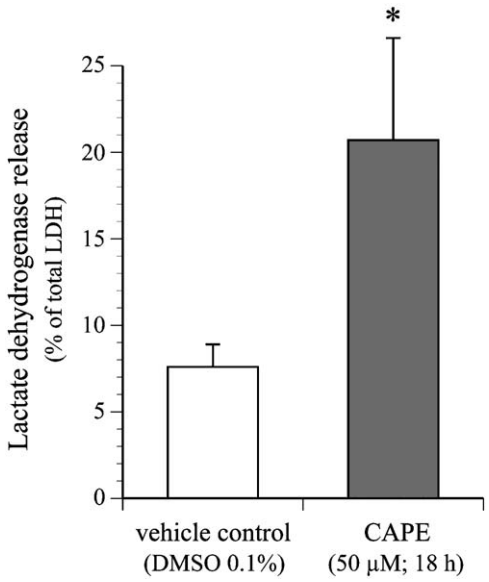

Fig. 3. CAPE induced cytotoxicity in C2C12 myotubes. Cytotoxicity was assessed by the release of lactate dehydrogenase (LDH) into the cell medium over an 18 h treatment with 50 μM CAPE or vehicle (0.1% DMSO). Released LDH was expressed as a % of total LDH. Data are presented as the mean of 3 experiments ± SEM. * Indicates a significant ($p \leq 0.05$) difference from the vehicle control group.

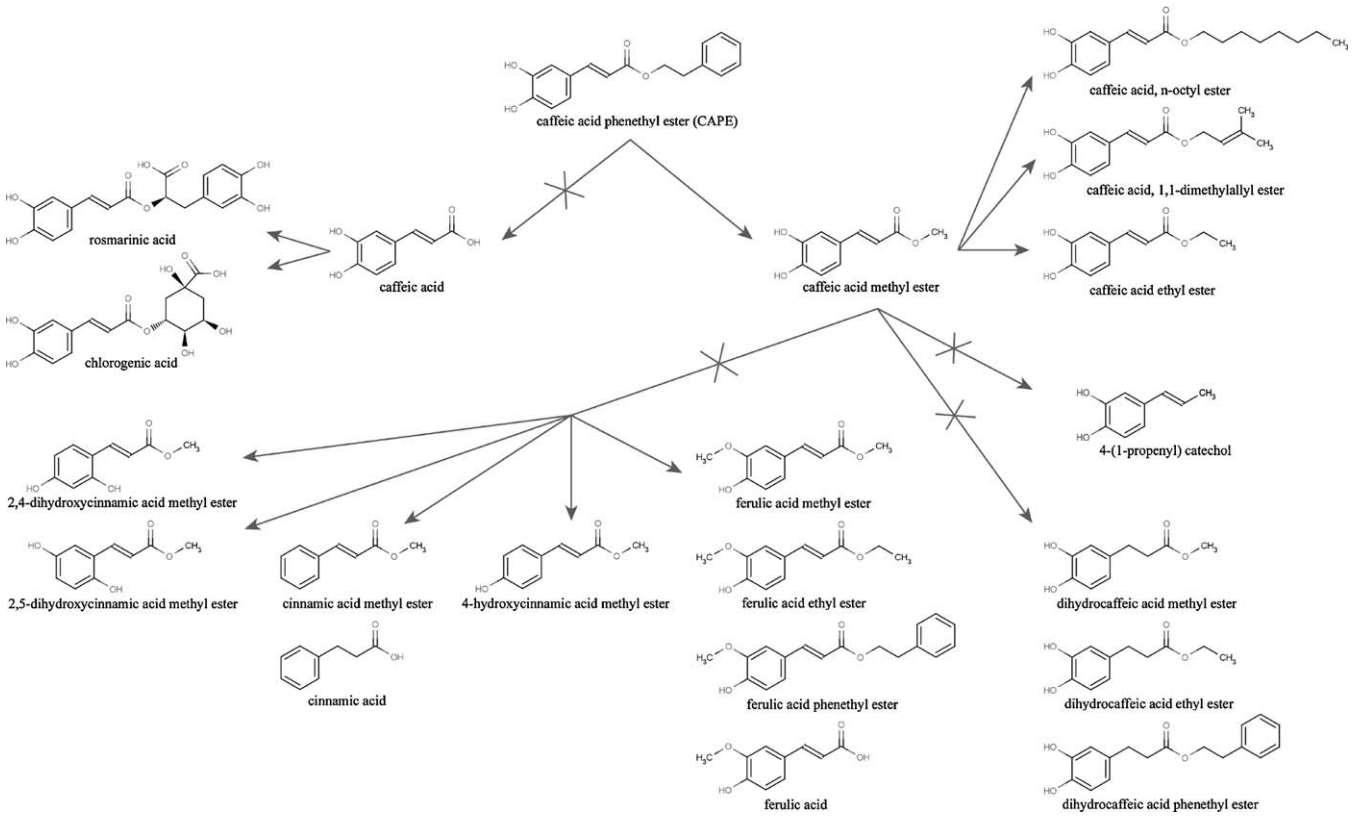

Fig. 4. Compounds selected to address specific structure–activity hypotheses. Compounds were initially selected based on a relation to CAPE. Further testing was performed on compounds more closely related to CAME. Each arrow represents a specific hypothesis. Arrows with crosses indicate that the respective derivatives, and any nested derivatives, are inactive.

3.4. Effect of caffeic acid derivatives on mitochondrial function and glucose uptake

In order to assess whether other compounds related to CAPE possess similar activities and to elucidate a structure–activity relationship, twenty compounds were tested for glucose-uptake stimulating activity and for mitochondrial uncoupling activity. These compounds are illustrated in Fig. 4, functionally grouped to address discrete structural hypotheses. Activities of these compounds, in addition to some physicochemical properties, are summarized in Table 1.

A first step consisted of testing the root compound, caffeic acid; it was found to be inactive in both assays. Next, a more closely related compound, CAME, was found to increase glucose uptake by 65% and to mildly uncouple oxidative phosphorylation by 7%. In light of this finding, other caffeic acid esters were tested, including CAEE, CAAE, and CAOE; all three were found to be active, increasing uptake by 72–230%, and uncoupling oxidative phosphorylation by 14–92%.

Working from the active CAME, four closely related esters differing only in the number or position of hydroxyl substituents around the phenolic ring were tested. All were found to be inactive in either assay. Similarly, ferulic acid methyl ester was inactive. Still working from CAME, a related compound missing the characteristic double bond of caffeic acid, caffeic acid dihydro methyl ester, was found inactive; similarly, other caffeic acid dihydro esters were inactive. The compound propenyl catechol, a truncated caffeic acid devoid of the carboxylic acid ester, was also inactive. Finally, some free caffeic acids (i.e. carboxyl substituted), including rosmarinic and chlorogenic acid, were found inactive.

3.5. Effect of active caffeic acid derivatives on cellular viability

The four newly identified active caffeic acid derivatives were tested for CAPE-like cytotoxicity. CAAE, CAAE, and CAOE all decreased viability by 3–16% (Table 1). CAME, however, did not affect the release of LDH, as compared to vehicle alone.

3.6. Relationship between uncoupling of oxidative phosphorylation and stimulation of glucose uptake

Of the 21 compounds in the test set, only the five caffeic acid esters were found to induce an uncoupling effect in isolated mitochondria. These five were also the only compounds to induce an important (>30%) enhancement of glucose uptake. A linear regression analysis of the 21 compounds supported that uncoupling activity, measured as an instantaneous effect, and stimulation of glucose-uptake following an 18 h treatment, were related activities (Fig. 5). However, the closeness of fit was slightly reduced by CAOE, equally potent to CAPE at stimulating glucose uptake but a less powerful uncoupler under the conditions used here.

3.7. Stimulation of the AMPK pathway by active caffeic acid derivatives

Western immunoblot analyses were performed in order to confirm that the four newly identified active caffeic acid derivatives activated the AMPK pathway, as has been reported for CAPE. The content of phosphorylated ACC, an effector of AMPK, was assessed in C2C12 cells treated with caffeic acid derivatives (50 μM) or with vehicle alone for 18 h. All four compounds and CAPE were found to induce a long-lived phosphorylation of ACC,

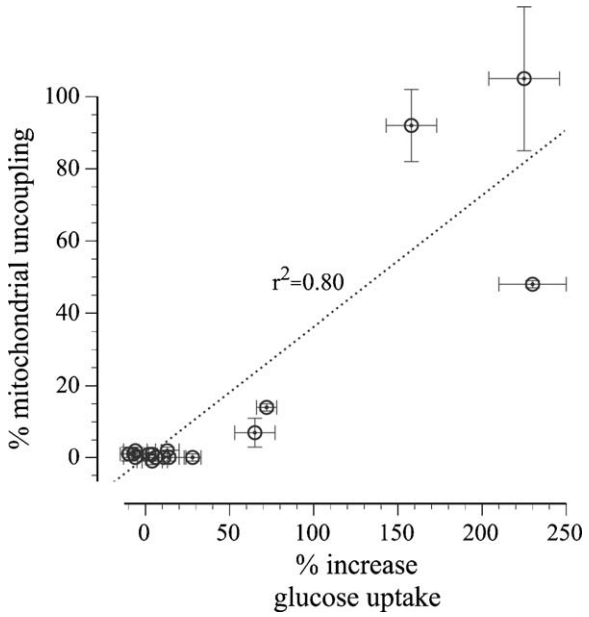

Fig. 5. The magnitude of stimulation of glucose uptake in C2C12 mytotubes induced following an 18 h treatment with caffeic acid derivatives is correlated to the magnitude of mitochondrial uncoupling activity induced by these compounds in isolated liver mitochondria. Data are presented as mean ± SEM.

although in all cases the content of phospho-ACC was inferior to that induced by the AMP mimetic AICAR (1 mM) applied to vehicle-control cells over the last 30 min of treatment (Fig. 6).

3.8. Relationship between lipophilicity and effect on mitochondrial function

An important physicochemical property that varied between the five active caffeic acid derivatives was lipophilicity. Non-linear regression analyses were therefore performed to assess whether lipophilicity was a predictor of activity. Lipophilicity, expressed as the log of the predicted octanol–water partition coefficient (P), was found to be well-related ($r^2 = 0.99$) to enhancement of glucose uptake by a sigmoidal dose–response function (Fig. 7A). Lipophilicity was also found to be well-related ($r^2 = 0.99$) to the decrease of cellular viability, again by a sigmoidal function (Fig. 7B). Finally, lipophilicity was well-related ($r^2 = 0.99$) to uncoupling over the log P range of 1.9–3.9, also by a sigmoidal function (Fig. 7C); CAOE, the most lipophilic compound, induced less uncoupling than CAPE or CAAE under the conditions used here, as noted above and as discussed below.

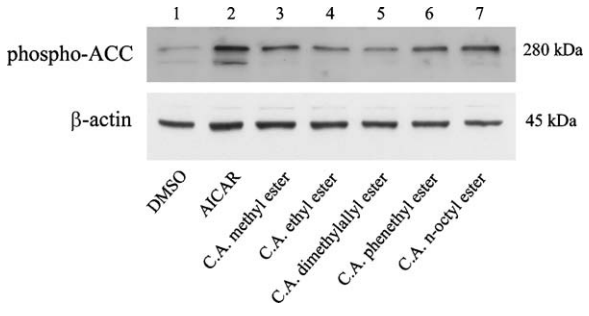

Fig. 6. Active caffeic acid derivatives increased phosphorylation of ACC, an effector of AMPK, in C2C12 myotubes. Shown are representative immunoblots of cells treated for 18 h with either vehicle (0.1% DMSO; lane 1) or 50 μM of the various compounds (lanes 3–7). The upper blot was probed with anti-phospho-ACC. The lower blot was probed with anti-β-actin as a control. AICAR (1 mM) applied acutely for the last 30 min of the treatment in vehicle-treated cells was used as positive control (lane 2) for the activation of the AMPK pathway.

4. Discussion

Derivatives of caffeic acid, a subtype of cinnamic acid, are widely distributed in the plant kingdom and are found in coffee beans, wheat, oat, and several fruits and vegetables. They typically occur not as free acids, but as esters, amides, and glycosides, or as dimers and other more complex forms [27]. These compounds as a family have received much attention in recent years and a variety of activities, including anti-bacterial, anti-cancer, anti-inflammatory, anti-atherosclerotic, anti-oxidant, immunomodulatory and neuroprotective, have been ascribed to them [28–42]. It has also been proposed that caffeic acid derivatives may possess anti-diabetic activities [43–46]. A particularly interesting member of this family is caffeic acid phenethyl ester (CAPE), best known as one of the main botanical components of honeybee propolis [47], a glue-like substance used in the making of beehives and which exhibits potent anti-microbial activity believed to contribute to the aseptic environment of the hive. CAPE has recently been observed to exhibit anti-diabetic activity in the form of potent AMPK-mediated stimulation of glucose uptake in skeletal muscle cells [17]. As AMPK is considered a key therapeutic target for metabolic diseases and current therapies for exploiting this target are limited, the present study was designed to probe the potential of CAPE and caffeic acid derivatives as novel activators of AMPK with glucose-uptake stimulating activity. Specifically, the aims of the study were to confirm the effect of CAPE, elucidate the mechanism by which it activates AMPK, identify other caffeic acid derivatives with similar anti-diabetic activity, elucidate the structural components and physicochemical properties essential to their activity, and evaluate the relationship between activity and cytotoxicity.

Lee et al. [17] have recently shown that, following a 1 h treatment, CAPE induces an AMPK-mediated stimulation of glucose uptake in L6 skeletal muscle cells that is comparable to the effect of 100 nM insulin. In the present study, we report that a treatment duration of 18 h at 50 μM resulted in more than a 3-fold increase in basal (non-insulin-stimulated) glucose uptake in C2C12 muscle cells, an effect approximately 6-fold that of 100 nM insulin applied acutely. This larger effect magnitude relative to the Lee et al. study [17] may be due to our use of a longer treatment duration and of a less insulin-responsive cell line, both factors permitting a better appreciation of the contribution of transcriptionally mediated effects rather than effects only at the level of translocation and activation of glucose transporters. Indeed, increased expression of effector proteins appears a likely explanation for effects surpassing those achieved acutely with a pharmacological dose of insulin and presumably representing the system's maximal capacity. If a long-lived translocation effect contributed to some of the observed stimulation, as may be suggested by the finding that the AMPK effector ACC remains phosphorylated at the end of the 18 h treatment, this contribution would be expected to be on the order of the acute effect of insulin since AMPK-induced transporter translocation is mediated by signaling events that converge with the insulin receptor pathway. Similarly as shown by Lee et al. [17], a contribution of the insulin receptor pathway to the effect of CAPE was excluded by the demonstration that there was no increase in the phosphorylation of Akt, a downstream marker involved in glucose transporter translocation, coinciding with the enhancement of glucose uptake (not shown). The exceptionally large increase in glucose uptake can therefore best be explained by other mechanisms attributed to AMPK, namely an increase in maximal capacity for glucose uptake [6,7,22,23].

To test the hypothesis that CAPE activates AMPK by disrupting mitochondrial function and producing metabolic stress, CAPE was applied to isolated rat liver mitochondria and its effects on oxygen consumption were monitored. Our results demonstrate that CAPE

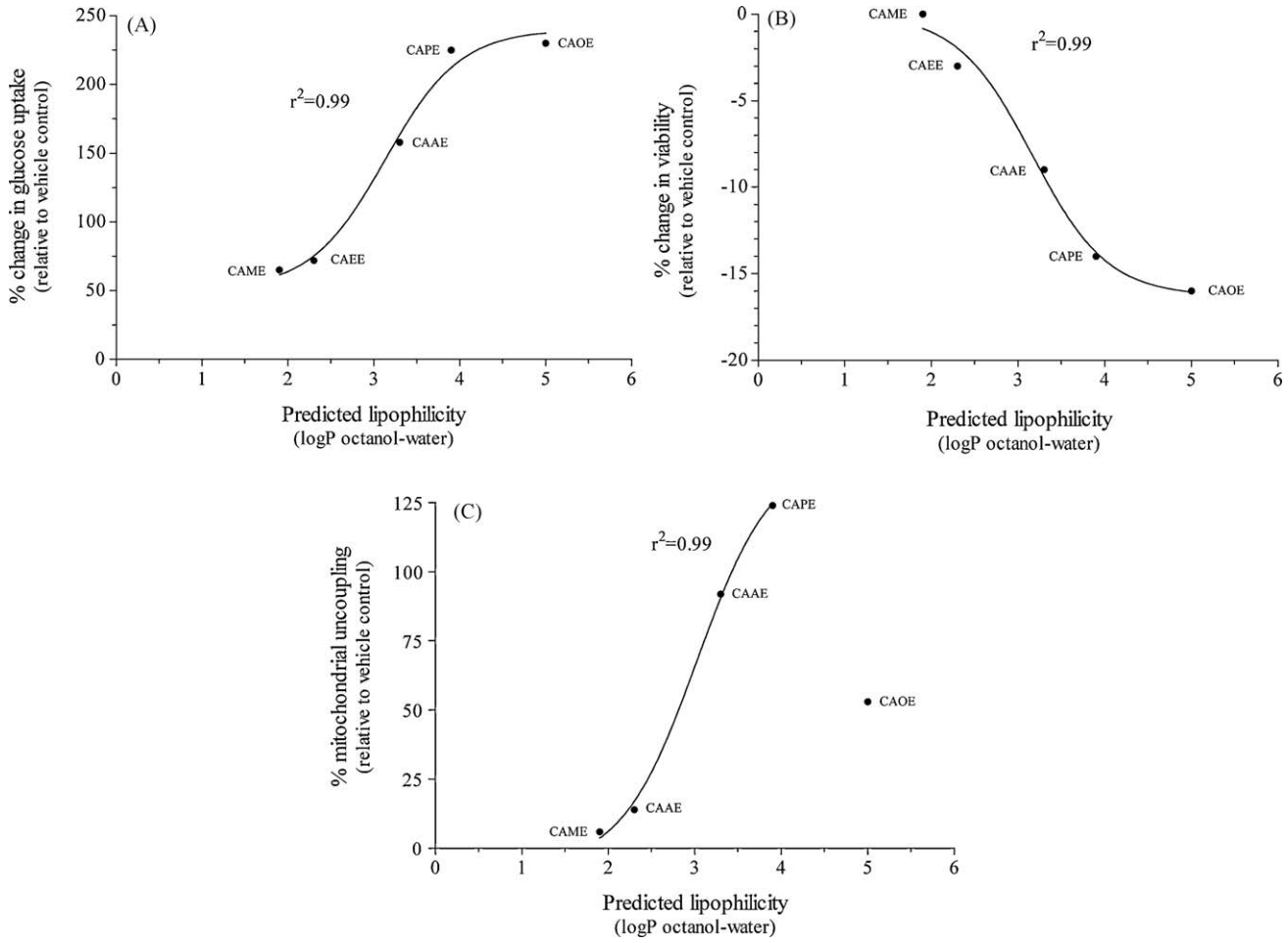

Fig. 7. The lipophilicity of active caffeic acid derivatives is a good predictor of their activity. The stimulation of muscle cell glucose uptake (A) as well as the negative impact on C2C12 viability (B) were well-related to estimated lipophilicity, expressed as log of the octanol–water partition coefficient (*P*). Mitochondrial uncoupling (C) was also well-related to lipophilicity, but only over a log *P* range of 1.9–3.9; the uncoupling activity of the most lipophilic compound CAOE was determined to be smaller than that of CAPE and CAAE and may have been underestimated due to concurrent inhibition of mitochondrial respiration.

at 50 μM induced complete uncoupling of liver mitochondria, whereby the rate of basal (i.e. State 4) oxygen consumption was immediately and irreversibly increased above that which can be achieved by ADP, and the addition of ADP had no further effect. These results will be further strengthened by assessing the effects of CAPE in skeletal muscle's two distinct mitochondrial populations. Because of the magnitude of CAPE's effect on mitochondrial function, its cytotoxicity was assessed by LDH assay in C2C12 cells; CAPE was observed to reduce viability by 14% following an 18 h treatment at 50 μM. Our finding that CAPE exhibits a potent uncoupling effect and can completely dissipate ATP synthetic capacity in isolated mitochondria concords with its use as an antimicrobial compound by plants and insects, and with other botanical components of propolis exhibiting uncoupling activity [16]. This finding is also in accord with the activation of AMPK being the result of a metabolic stress [8,48]. Although the uncoupling-type disruption of mitochondrial function observed here is different from the inhibition of complex I of the electron transport chain that is induced by Metformin [49,50], the proposed mechanism of action by which CAPE indirectly induces the activation of AMPK is nevertheless analogous to that of Metformin.

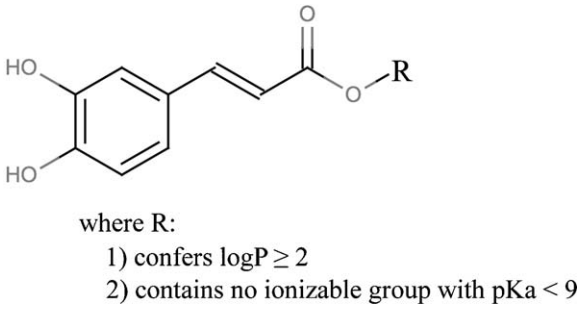

Fig. 8. Structural constraints for bioactive caffeic acid derivatives. Activity requires that: (1) the catechol moiety be intact; (2) the double bond between the first and second carbons of the side chain be present; (3) the carboxylic acid ester be present; (4) the side chain not contain strongly ionizable groups. Beyond these constraints, the side chain can be composed of various structures, modulating activity in accordance with the lipophilicity that they confer upon the compound; within the log *P* range of 2–5, activity is linearly related to lipophilicity.

Caffeic acid esters closely related to CAPE were tested for glucose uptake stimulating activity and for mitochondrial uncoupling activity. These included caffeic acid methyl ester (CAME), caffeic acid ethyl ester (CAEE), caffeic acid diallyl ester (CAAE), and caffeic acid n-octyl ester (CAOE). All four were found to potently increase basal uptake by 65–230% when applied at 50 μM for 18 h. These same four were also found to uncouple mitochondria by 7–92%. Finally, these compounds were tested for cytoxicity and it was found that, with the exception of CAME, they reduced viability by 3–16%. Stimulation of glucose uptake and mitochondrial uncoupling have never been attributed to these well-known compounds, although CAOE, like CAPE has received attention for its anticancer

activity [51–53], a property that could be related to the effect on mitochondrial function and the subsequent activation of AMPK, observed herein; indeed, cellular proliferation is one of the many synthetic processes that are acutely inhibited by AMPK [54].

Sixteen other related compounds were selected to address specific structure–activity hypotheses. Of these, none exhibited uncoupling activity nor significantly stimulated muscle cell glucose uptake. The finding that caffeic acid and other related free acids were inactive suggests that a carboxyl group and perhaps other strongly ionizable substituents are incompatible with activity, possibly due to decreased membrane permeability of ionized compounds. The requirement for an intact catechol moiety was revealed by an absence of activity in compounds related to CAME but differing in the number or position of hydroxyl substituents. Similarly, absence of the caffeic acid double bond or of the carboxylic acid ester was also found to abolish activity. The structural elements occurring beyond the ester can therefore be considered as a single substituent not essential to activity but whose nature modulates activity. As such, it can be predicted that caffeic acid esters composed of a large variety of "substituents" not containing a strongly ionizable group should be active. This is summarized in Fig. 8.

Uncoupling is defined herein as an increase in respiration (i.e. substrate oxidation) with no commensurate increase in the synthesis of ATP through oxidative phosphorylation. This increase in oxygen consumption is a reflection of an increase in the pumping rate of protons out of the mitochondrial matrix to compensate for an induced proton influx or "leak". The leak and the compensatory pumping of protons therefore amount to a futile metabolic cycle. The increase in oxygen consumption represents a portion of mitochondrial respiratory capacity diverted to countering the leak, and therefore a corresponding decrease in the maximal rate of ATP synthesis. AMPK can be expected to be activated as a result of the increased work performed pumping protons and of insufficient residual mitochondrial capacity to meet the cell's energy needs (i.e. metabolic stress). An uncoupling effect can be induced by any of a number of protonophoric mechanisms, the best studied of which involves the shuttling of protons across the inner mitochondrial membrane by lipophilic weak acids that diffuse into the mitochondrial matrix in neutral form, release a proton, and diffuse back to the mitochondrial intermembrane space (IMS) in ionized form [55,56]. Protons can be similarly shuttled by certain fatty acids that intercalate inner mitochondrial membrane phospholipids and exist there in both neutral and ionized forms [56,57]. The caffeic acid derivatives exhibiting uncoupling activity are unlikely to be acting as proton shuttles since, by virtue of their pK_a on the order of 9.2, they are expected to exist predominantly in the neutral form at mitochondrial matrix pH (approximately 8.0), and almost exclusively in this form at mitochondrial IMS pH (approximately 7.4). These compounds may therefore be indirect protonophores rather than shuttles. One possibility is that the compounds interact with a transmembrane protein that can increase proton conductance. Such proteins include transporters like the adenosine nucleotide transporter, the aspartate/glutamate transporter, and the dicarboxylate carrier, either alone or as part of the mitochondrial permeability transition pore (MPTP) or another complex [56,58,59]. The potential interaction of caffeic acid derivatives with a protein is supported by the observation that activity is conferred only by a very specific structure (i.e. the caffeic acid moiety) and that small deviations in this structure abolish activity, as discussed above. In contrast, proton shuttles are not subject to such severe structural constraints, but rather to constraints at the level of their physicochemical properties [55,56]. The interaction of resveratrol and of quercetin with ATP synthase, recently elucidated by crystallography [15], constitutes a precedent for binding of naturally occurring small phenolic compounds to protein components of the oxidative phosphorylation system. Interaction with a protein of the MPTP, and subsequent proton conductance through the pore, has been proposed by others to explain the uncoupling activity of curcumin [60,61], a compound closely related to caffeic acid derivatives. Based on the structural constraints for activity, it can be speculated that the proposed interaction is mediated by the two hydroxyl substituents of the catechol moiety in addition to the carboxylic acid ester, that the ester must be coplanar with the phenolic ring by virtue of the double bond, and that the interaction occurs on the matrix side of the inner mitochondrial membrane, inaccessible to negatively charged compounds.

The results demonstrate that whereas an intact caffeic acid moiety is essential for activity, the "substituent", or structure occurring after the carboxylic acid ester, can assume a variety of forms. However, despite not being subject to stringent structural constraints, this portion can nevertheless greatly affect the properties of the entire compound. From a physicochemical perspective, the most important difference between the five active compounds tested here is their lipophilicity as the compounds span a predicted log *P* range of 1.9–5.0. Interestingly, this property was found to be a strong predictor of activity, with goodness of fit coefficients (r^2) of $\geq$0.99 observed between log *P* and both glucose uptake and cytotoxicity when these relationships were modelled by sigmoidal dose–response functions. It can therefore be expected that the activity of other active caffeic acid esters will be found to be also predicted by lipophilicity. It must be noted that a strong sigmoidal relationship between lipophilicity and mitochondrial uncoupling activity was only observed within the log *P* range of 1.9–3.9. The most lipophilic compound, CAOE, exhibited less uncoupling activity than either CAPE or CAAE. Modest activity in highly lipophilic compounds could be explained by a decrease in effective concentration due to the phenomenon of membrane retention. However, in the present case, it may be explained by an underestimation of uncoupling activity, suggested by a significantly greater decrease in residual mitochondrial capacity than can be accounted for by the uncoupling effect alone. Such concurrent inhibition of respiration has been suggested by others to be due to inhibition of the mitochondrial transport of succinate [62–64], the substrate used in our isolated mitochondrial preparations. Interestingly, CAPE exhibits a similar effect at concentrations greater than the 50 μM used in the present study (data not shown). In whole cells, mitochondria oxidize other substrates in addition to succinate, and therefore respiration would not be expected to be severely compromised by a partial inhibition of succinate transport. It is therefore possible that the true uncoupling activity of CAOE may be similar to that of CAPE, just as the glucose-uptake stimulating activity of these compounds is closely matched. In this case, the relationship between lipophilicity and uncoupling activity would mirror the observed relationship between lipophilicity and stimulation of glucose uptake.

While the disruption of mitochondrial function through which caffeic acid derivatives promote the activation of AMPK is slightly different from the inhibitory-type disruption induced by the biguanides, as discussed above, both mechanisms can potentially cause lactic acidosis. Therefore, as is the case for the biguanides, the safety of caffeic acid derivatives can only be maximized at the cost of activity. However, results indicate that some of the active caffeic acid derivatives identified here exhibit only a small potential for toxicity while still inducing significant stimulation of glucose uptake and prolonged phosphorylation of ACC. This is especially true of CAME as its small uncoupling effect (7% at 50 μM) translates into a useful increase in glucose uptake (65% at 50 μM; more than 1.5-fold the effect of insulin), without negatively impacting cell viability. However, even the more powerful compounds of the test group that completely or almost

completely compromise mitochondrial ATP synthesis only reduce viability by up to 16%. One way to reconcile these findings is to suggest that their effect on mitochondria is short-lived. It is indeed known that phenolic compounds with hydroxyl substituents can undergo rapid glucuronidation [65]. In the case of active caffeic acid derivatives, such glucuronidation would likely render compounds inactive toward mitochondrial uncoupling. Whereas such short-lived mitochondrial activity would suggest equally short-lived metabolic stress and activation of AMPK, the downstream effects of AMPK, including its effects on gene expression, are longed-lived. It may therefore not be necessary nor desirable to prolong metabolic stress.

The very promising ratio of activity to cytotoxicity of CAME warrants further study into the potential of this compound and of other related derivatives as treatments for insulin resistance and suggests that activation of AMPK through disruption of mitochondrial function need not have a narrow margin of safety.

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