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Uncoupling of oxidative phosphorylation by curcumin: Implication of its cellular mechanism of action

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ABSTRACT

Curcumin is a phytochemical isolated from the rhizome of turmeric. Recent reports have shown curcumin to have antioxidant, anti-inflammatory and anti-tumor properties as well as affecting the 5′-AMP activated protein kinase (AMPK), mTOR and STAT-3 signaling pathways. We provide evidence that curcumin acts as an uncoupler. Well-established biochemical techniques were performed on isolated rat liver mitochondria in measuring oxygen consumption, F_0F_1-ATPase activity and ATP biosynthesis. Curcumin displays all the characteristics typical of classical uncouplers like fccP and 2,4-dinitrophenol. In addition, at concentrations higher than 50 μM, curcumin was found to inhibit mitochondrial respiration which is a characteristic feature of inhibitory uncouplers. As a protonophoric uncoupler and as an activator of F_0F_1-ATPase, curcumin causes a decrease in ATP biosynthesis in rat liver mitochondria. The resulting change in ATP:AMP could disrupt the phosphorylation status of the cell; this provides a possible mechanism for its activation of AMPK and its downstream mTOR and STAT-3 signaling.

Introduction

Curcumin (CUR) is a yellow pigment, isolated from the rhizome of *Curcuma longa* (turmeric). Turmeric is widely used in several Asian countries as a dietary spice in foods, in traditional medicines and pharmaceutical industries [1]. It has been reported that CUR induces apoptosis in cancer cells [1–3]. This explains the current interest of its anti-cancer properties and its use in clinical trials for various types of cancers [4]. However, the mechanism of induction of apoptosis remains obscure and several mechanisms have been proposed, i.e. via mitochondrial hyperpolarization and mitochondrial DNA damage [3], via membrane protein thiol oxidation and hence, opening of the mitochondrial permeability transition pore (PTP) [5] and via a mitochondrion- and caspase-independent pathway [6].

In this study, we provide evidence that CUR at 20 μM and 50 μM is an uncoupler which increases state 4 respiration in isolated rat liver mitochondria. This phenomenon has previously been shown with fluoride derivatives of curcumin, but not curcumin itself [7]. The stimulated state 4 respiration is reversed by concentrations of CUR higher than 50 μM and by 6-ketocholestanol (6-KCh), a known re-coupler [8–10]. Our study showed that CUR caused a marked decrease in ATP biosynthesis from the oxidation of various respiratory substrates. It also stimulates F_0F_1-ATPase of rat liver mitochondria. As a protonophore, CUR induced swelling of valinomycin-treated rat liver mitochondria incubated in a hyposmotic medium of potassium acetate [11].

As CUR decreases ATP biosynthesis, the change in phosphorylation status in the cell would have an impact on the phosphorylation-dependent cell signaling. One such pathway is regulated by the master energy sensor, 5′-AMP protein kinase (AMPK) which can be activated by an increased AMP/ATP ratio. CUR has been shown to activate AMPK but its mechanism remains unknown [12,13]. Here, we propose that activation of AMPK by CUR is mediated via its protonophoric-uncoupling action and its activation of F_0F_1-ATPase. The combined net effect is a fall in cellular ATP resulting in a change in the AMP:ATP ratio which induces AMPK.

Materials and methods

Chemicals

All materials were purchased from Sigma–Aldrich Chemical Co., St. Louis, MO, USA.

Animal studies and cell culture

Care and maintenance of laboratory animals were in accordance with the guidelines of the Institutional Animal Care and Use

Abbreviations: ANT, adenine nucleotide translocase; CATR, carboxyatractyloside; CsA, cyclosporin A; CUR, curcumin; DNP, 2,4-dinitrophenol; fccP, carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone; 6-KCh, 6-ketocholestanol; PTP, permeability transition pore.

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Committee (IACUC) of National University of Singapore. Adult male Wister rats of about 200 g were used. The HepG2 cell line was from ATCC, originally derived from the liver tissue of a 15-year-old Caucasian male with hepatocellular carcinoma.

Isolation of mitochondria

(a) From rat liver and brain. Mitochondria were isolated from the liver and brain of male Wistar rats by differential centrifugation using the procedure reported previously from our laboratory [14]. The freshly isolated mitochondria were used immediately for measurement of (a) oxygen consumption by the oxygraph (b) ATP biosynthesis by the luciferin–luciferase assay and (c) mitochondrial swelling, or stored at −80 °C for the determination of activities of F_0F_1-ATPase and respiratory complex IV.

(b) From HepG2 cells. Mitochondria were isolated from HepG2 cells using the procedures as reported in [15]. All the steps were carried out at 4 °C. Cells from nine T-75 flasks were trypsinized, centrifuged at 600 *g* for 5 min and the pellet was suspended in 1 ml of solution A which contained 250 mM sucrose, 20 mM Hepes, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, and 1 mM dithiothreitol. This was followed by homogenization with 20 up-and-down strokes in a glass homogenizer. The homogenate was then centrifuged at 800 *g* for 10 min. The supernatant was collected and centrifuged at 10,000 *g* for 15 min. The pellet was suspended in 1 ml of solution A and re-centrifuged under the same conditions and finally re-suspended in 200 μl of solution A and kept at −80 °C for the determination of F_0F_1-ATPase activity.

Polarographic measurements

A Clark-type oxygen electrode (Hansatech, UK) was used to measure the rate of oxygen uptake. 0.3 mg of freshly prepared rat liver mitochondria was added to 2 ml of MiR05 respiratory medium [16] at 30 °C. State 4 respiration was started with 5 mM each of glutamate and malate (glu/mal) followed by 20–500 μM curcumin. To induce state 3 respiration, 125 μM of ADP was added 10 min after the introduction of the respiratory substrates. The sequence of addition of fccP, carboxyatractyloside (CATR), cyclosporin A (CsA), oligomycin and 6-kCh was as described in the individual experiments.

Purification of F_1-ATPase from rat liver mitochondria

The partial purification of F_1-ATPase involved chloroform extraction and separation by a PD-10 column packed with Sephadex G25 of medium grade [17]. The fractions containing F_1-ATPase activity were pooled, lyophilized and stored at −20 °C.

Determination of mitochondrial F_0F_1-ATPase and F_1-ATPase activity

The F_0F_1- and F_1-ATPase activities were measured by a coupled assay using lactate dehydrogenase and pyruvate kinase [18]. The freeze–thawed extract of mitochondria (0.1 mg protein) or partially purified F_1-ATPase (4.7 μg protein) was pre-incubated with 10–100 μM CUR, resveratrol (100 μM) or oligomycin (10 μg/ml) for 6 min at 37 °C. The decrease in NADH was measured every 11 s for 10 min at 340 nm in an absorbance microplate reader.

ATP biosynthesis in isolated rat liver mitochondria

The luciferin–luciferase reaction as reported previously from our laboratory was used [19,20].

Measurement of intracellular ATP in HepG2 cells

Cells (100,000) plated on a 24-well plate and grown overnight were examined the next day under the microscope to ensure adherence to the culture plate. The medium was then discarded, followed by the addition of 70 μM CUR prepared in DMEM. After 24 h, the cells were washed with ice-cold PBS before the addition of 0.5 ml of ice cold deionized water into each well followed by sonication for 3 min. The cells were scraped from the wells using a pipette tip, transferred to pre-cooled Eppendorf tubes, boiled for 3 min and centrifuged at 15,000 rpm for 10 min at 4 °C. Intracellular ATP was measured based on the luciferin–luciferase reaction as described in [20].

Measurement of mitochondrial swelling in a hyposmotic medium of potassium acetate

The identity of a protonophore is based on the principles of mitochondrial swelling as detailed by Nicholls [11]. The assay was carried out using the procedures as described by Mingatto et al. [21] with some modifications. 10 μl of freshly prepared rat liver mitochondria (0.45 mg protein) were incubated at 37 °C for 3 min in 190 μl of hyposmotic potassium acetate solution containing 54 mM potassium acetate, 5 mM Hepes, pH 7.1, 0.1 mM EGTA, 0.2 mM EDTA, 0.1 mM sodium azide, 1 mg/ml BSA, 15 μM CATR, 1 μM antimycin A and 0.3 mM propanolol. Valinomycin (1 μM) and different concentrations of CUR were then added followed by the measurement of mitochondrial swelling from the change in absorbance at 540 nm in an absorbance microplate reader (SpectraMax 190 from Molecular Devices Corporation, USA).

Determination of complex IV activity in rat liver mitochondria

A polarographic assay of Complex IV (cytochrome *c* oxidase) as described by Brautigan et al. [22] and outlined by Varela et al. [23] was used with a slight modification. The reaction was carried out at 30 °C in 2 ml of a standard respiratory medium containing 130 mM sucrose, 50 mM KCl, 5 mM $MgCl_2$, 5 mM KH_2PO_4, 50 μM EDTA and 5 mM Hepes, pH7.4, supplemented with 2 μM rotenone, 10 μM oxidized cytochrome *c* and 0.02% Triton X-100. Freeze-thawed rat liver mitochondria (0.5 mg protein) and 10 or 100 μM CUR or DMSO (control) were added and allowed to incubate for 3 min before the initiation of reaction by the addition of 0.25 mM TMPD + 5 mM ascorbate.

Results

Curcumin on state 4 and state 3 respiration

State 4 respiration of rat liver mitochondria supported by 5 mM glu/mal was stimulated by 20 μM and 50 μM CUR. However above 50 μM, although an overall stimulation was still observed, the degree of stimulation decreased in a concentration-dependent manner (Fig. 1A).

The ADP-stimulated state 3 respiration was also inhibited in a concentration-dependent manner (Fig. 1B). These results showed that CUR acts as an uncoupler at 20 and 50 μM. However, at higher concentrations, CUR progressively inhibited mitochondrial respiration.

Curcumin on fccP-stimulated state 4 respiration

The stimulation of state 4 respiration by 10 μM fccP was compromised by 500 μM CUR (Fig. 1C). A similar inhibitory action

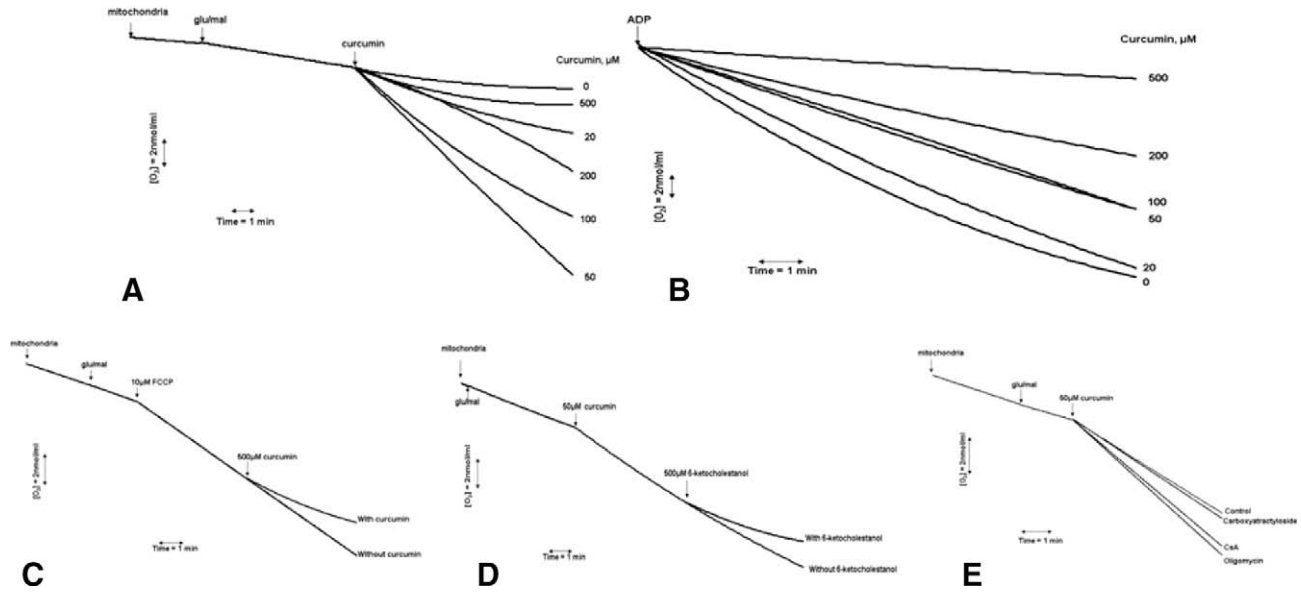

Fig. 1. Polarographic measurements. Each set of data is representative of three separate experiments. The quality of mitochondria was assessed routinely and the respiratory control ratio (RCR) obtained was generally between 3 and 4. (A) Curcumin on state 4 respiration. (B) Curcumin on ADP-stimulated state 3 respiration. (C) Curcumin on fccP-stimulated state 4 respiration. (D) 6-Ketocholestanol on curcumin-stimulated state 4 respiration. (E) Cyclosporin A, oligomycin, and carboxyatractyloside on curcumin-induced state 4 respiration. Isolated rat liver mitochondria were pre-incubated with 5 μM CATR, 1 μM CsA or 20 μg oligomycin/ml before the addition of 5 mM glu/mal.

was observed with 10 μM rotenone, a complex I inhibitor (data not shown).

Effects of 6-KCh, CATR, CsA, and oligomycin on curcumin-stimulated state 4 respiration

State 4 respiration stimulated by 50 μM CUR was reversed by 500 μM 6-KCh (Fig. 1D). A similar action of 6-KCh was also observed on state 4 respiration induced by fccP (data not shown). Uncoupling agents such as fccP, cccP and fluoride curcumin derivatives, were reported to be likewise inhibited by 6-KCh [7–9].

Uncoupling can also result by the opening of the PTP. To ensure that the increase in oxygen consumption during state 4 respiration induced by CUR was not so mediated, CsA, a PTP inhibitor was employed. In addition, oligomycin, and CATR were also examined under the same experimental conditions to rule out the possibility of the involvement of F_0F_1-ATPase and/or adenine nucleotide transporter (ANT). This is because uncoupler-stimulated ATPase has been reported [24] and the activation of F_0F_1-ATPase by CUR could increase ADP, a nucleotide known to close the PTP [25,26]. All the above three compounds, at the concentrations used, did not inhibit the stimulated state 4 respiration by CUR (Fig. 1E).

Effect of curcumin on F_0F_1-ATPase and partially purified F_1-ATPase activity

Curcumin activated F_0F_1-ATPase of rat liver mitochondria in a concentration-dependent manner as shown (Fig. 2A). Activation of rat liver mitochondrial ATPase is a feature common to all protonophoric uncouplers [27]. However, an inhibition was observed when rat brain mitochondria were used (Fig. 2B) and this is in agreement with the data of Zheng and Ramirez [28]. Little to no effect was observed with mitochondria isolated from HepG2 (Fig. 2C). The anomalous effects of CUR on the mitochondrial ATPases from different sources shown in our study concurred with the reports of Hayashi et al. [29].

CUR had no effect on partially purified F_1-ATPase prepared from rat liver mitochondria (Fig. 2D). Resveratrol (100 μM), a specific inhibitor of F_1-ATPase [28] decreased its activity by 70% while no effect was observed with 10 μg/ml oligomycin, an inhibitor of F_0-ATPase (data not shown).

Curcumin decreased ATP biosynthesis in isolated rat liver mitochondria

The rate of ATP biosynthesis from all respiratory substrates was decreased in the presence of CUR in a concentration-dependent manner (Fig. 3A); the degree of inhibition was most pronounced with TMPD/ascorbate. This inhibition could be due to the uncoupling and/or activation of F_0F_1-ATPase by CUR. In our study, intracellular ATP measured in HepG2 cells exposed to 70 μM CUR was significantly reduced to 34.42% ± 2.37 of the control (for $N = 3$ and $p < 0.005$). A concentration-dependent decrease in intracellular ATP was also reported when osteoblasts were treated with CUR [30].

Protonophoric action of curcumin on mitochondrial swelling

CUR between 10 and 100 μM induced a concentration-dependent swelling in rat liver mitochondria incubated in a hyposmotic solution of potassium acetate (Fig. 3B). This phenomenon is a characteristic feature of protonophores [11].

Curcumin does not affect respiratory complex IV activity

As 10 μM of CUR showed the greatest inhibitory effect on ATP biosynthesis from TMPD/ascorbate compared to the other respiratory substrates (Fig. 3A), complex IV activity was measured to examine if CUR inhibits electron transport through cytochrome oxidase. CUR at 10 and 100 μM did not inhibit complex IV activity (data not shown).

Discussion

Inhibitory uncouplers are chemicals that satisfy most if not all the criteria for uncouplers at low concentrations, but at higher con-

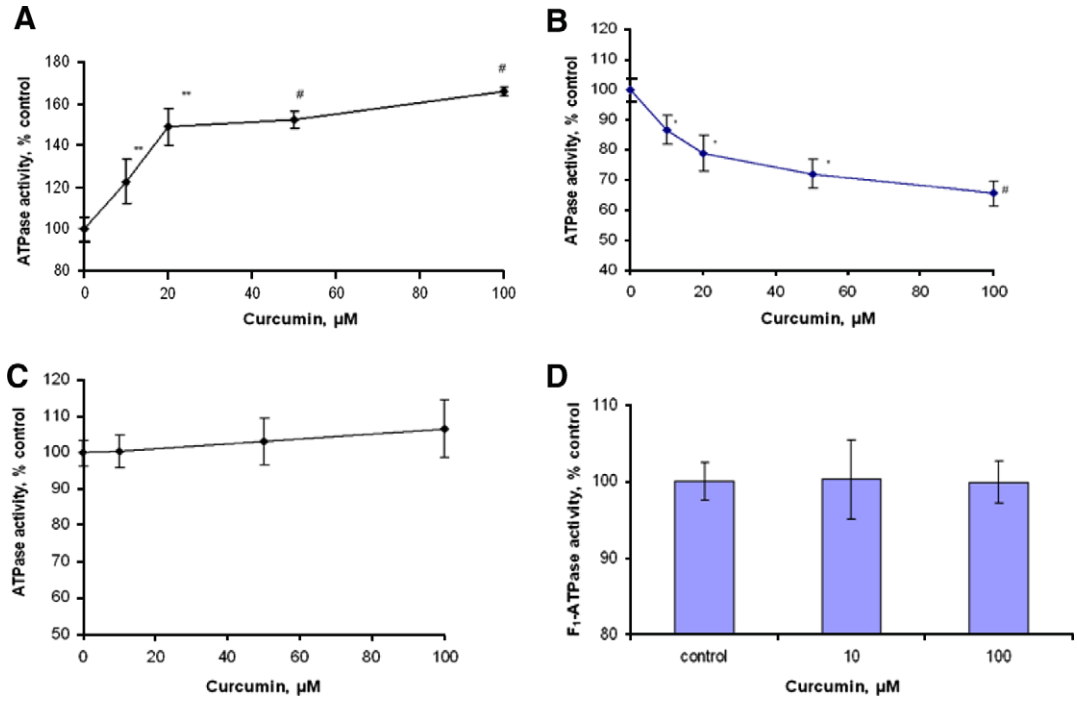

Fig. 2. Effect of curcumin on F_0F_1-ATPase (A–C) and F_1-ATPase (D) isolated from (A) rat liver mitochondria. (B) Rat brain mitochondria. (C) HepG2 mitochondria. (D) Rat liver mitochondria. The F_0F_1-ATPase activities in controls, expressed in nmol NADH/min/mg protein were, respectively, 40.92 ± 2.35, 24.53 ± 0.97, and 15.50 ± 0.55 for (A)–(C) above. These results were expressed as % of control of the means ± SD for $N = 3$; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{\#}p < 0.005$.

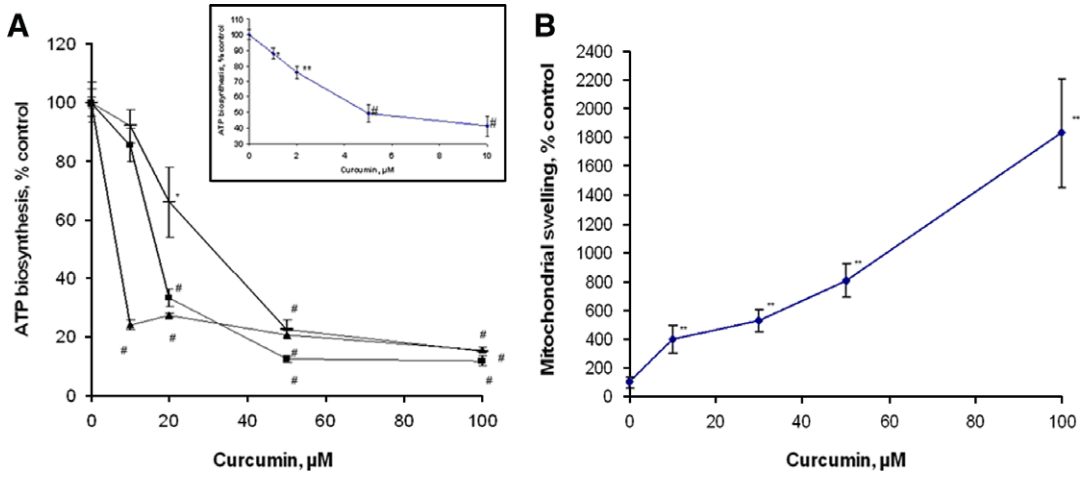

Fig. 3. (A) Effect of curcumin on ATP biosynthesis from the oxidation of 5 mM each glu/mal (trace —), 10 mM succinate + 5 μM rotenone (trace ■), and 1 mM TMPD + 5 mM ascorbate + 2 μg/ml antimycin A (trace ▲). The results were expressed as % of control of the means ± SD. $^{*}p < 0.05$; $^{\#}p < 0.005$ where $N = 3$. *Inset:* Curcumin as low as 1 μM caused a significant reduction in ATP biosynthesis from TMPD/ascorbate (complex IV substrates). (B) Curcumin induced swelling on valinomycin-treated mitochondria. The results were expressed as % of control of the means ± SD. $^{**}p < 0.01$ where $N = 3$.

centrations they act as inhibitors of electron transport [31]. These inhibitory uncouplers are generally substituted phenols whose action depends partially on the conformational changes in their binding niche in the cytochrome bc_1 complex [32]. Data presented in this study suggest that CUR could be so categorized because of its stimulation of state 4 respiration i.e. uncoupler-stimulated respiration at low micromolar concentration. At concentrations >50 μM, curcumin decreased both ADP-stimulated state 3 respiration and state 4 respiration induced by fccP and by itself at <50 μM, suggesting that it is a respiratory inhibitor. The uncoupling action of CUR was reversed by 6-KCh, a known re-coupler whose re-coupling mechanism is not established although it was proposed to alter the protein-mediated transport of such uncouplers [9]. If this is indeed the case, it is possible that CUR may target or bind to the protein that mediates the transport of protons. 6-KCh has been shown to reverse the effect of well known uncouplers like fccP and cccP [8,9].

With uncoupling, ATP biosynthesis from all respiratory substrates tested was compromised at 20 μM CUR. A significant degree of inhibition at 1 μM CUR was only demonstrated with TMPD/ascorbate as respiratory substrates. However, the intense yellow coloration of CUR, even at 1 μM precluded the use of the conventional spectrophotometric assay for measuring cytochrome oxidase activity. The alternative polarographic assay showed that at 10 and 100 μM CUR, complex IV activity was not affected which led us to conclude that the protonophoric action and possibly not

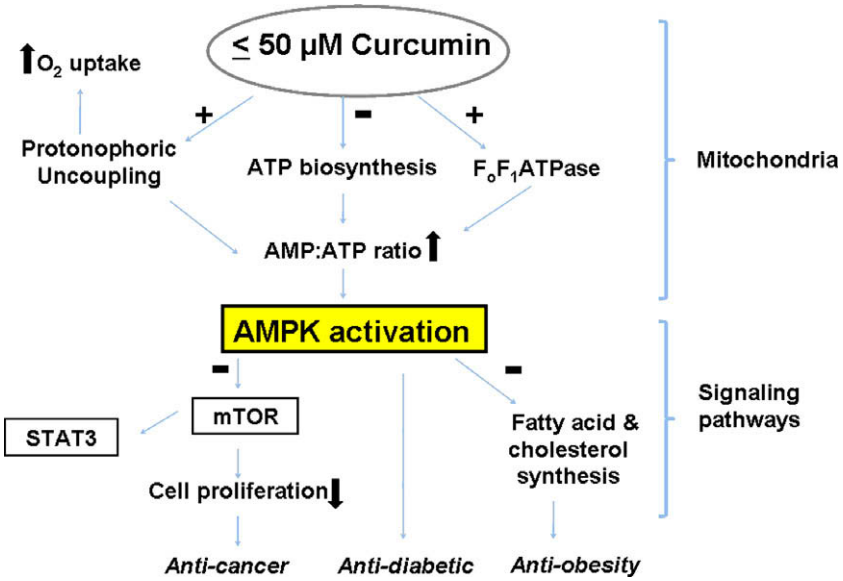

Fig. 4. Integrating the primary actions of curcumin on mitochondria with its proposed secondary effects on the downstream signaling via AMPK activation.

electron transport was responsible for the significant decrease in ATP observed. Unfortunately, it was not possible to confirm that uncoupling occurred at 1 μM CUR by polarographic analysis because of the lack of sensitivity of the oxygraph.

The protonophoric property of CUR was substantiated by its induction of swelling of valinomycin-treated rat liver mitochondria incubated in a hyposmotic solution potassium acetate [11]. The protonophoric-uncoupling mechanism of CUR could be reinforced by its activation of F_0F_1-ATPase activity which resulted in a fall in cellular ATP. However, CUR had no effect on partially purified F_1-ATPase suggesting that interaction of CUR was possibly via or with Fo-ATPase. In fact, it was reported that the stimulation of oligomycin-sensitive ATPase activity (in other words, F_0-ATPase) is a feature common to all protonophoric uncouplers [27]. A significant activation was observed at 10 μM CUR on F_0F_1-ATPase from rat liver mitochondria. In contrast, mitochondrial F_0F_1-ATPase from rat brain was inhibited and that from HepG2 (hepatocellular carcinoma) cells was not affected by CUR. This corroborates the report that uncoupler-stimulated ATPase was deficient in mitochondria isolated from hepatomas and ascites tumor cells [24]. The anomalous effects of CUR (i.e. activation/inhibition/no effects) on F_0F_1-ATPase from different sources as mentioned above were also reported for the classical uncoupler DNP [29]; the differential mechanisms of action remain unknown.

The physico-chemical properties of CUR contribute to its protonophoric-uncoupling activity. CUR has a dissociable proton with a moderate pK_{a2} of 7.97 [7]; it was shown that compounds with pK_a values between 4.3 and 8.5 can function as uncouplers [33]. Its partition coefficient expressed as $\log P^N_{oct}$ of 3.24 [7] would mean that CUR is sufficiently lipophilic to pass through membranes and function as an uncoupler, as we have established above.

This study highlights a hitherto unrecognized but significant action of CUR on mitochondrial oxidative phosphorylation which could explain its varied cellular effects as an anti-cancer, anti-diabetic and anti-obesity agent. A summary of the action of CUR on mitochondrial functions demonstrated in this study to explain its secondary effects on three signaling pathways, namely AMPK, mTOR and STAT-3 is shown in Fig. 4. A decrease in intracellular ATP due to uncoupling and/or its activation of F_0F_1-ATPase by CUR could explain the inhibition of the phosphorylation of STAT-3 at residue Y705 in a tumor cell line [34]. Likewise, the AMPK pathway can be activated by the increase in the AMP:ATP ratio. Other uncouplers like fccP have been shown to activate AMPK [35]. Activated AMPK in turn inhibits mTOR signaling and therefore inhibits proliferation of cancer cells which explains its potential in anti-cancer therapy [4]. mTOR signaling has been shown to phosphorylate STAT-3 at residue S727 [36,37]. Therefore, activation of AMPK and its inhibition of mTOR would decrease S727 phosphorylation of STAT-3 as shown by Ghosh et al. [38]. We have also found that 50 μM CUR decreased constitutively phosphorylated STAT-3 at residue S727 in HepG2 cells after a 2-h exposure but has no effect on Y705 on STAT (data not shown). Thus we propose that the protonophoric-uncoupling action of CUR could lead to the secondary activation of AMPK which is a well-known sensor of the status of cellular energy. AMPK in turn inhibits mTOR signaling which could decrease the phosphorylation STAT-3 at S727.

Besides being an anti-cancer agent as explained above via the AMPK/mTOR pathway, activated AMPK can also inhibit the synthesis of fatty acid and cholesterol [39] which explains its anti-obesity action. The body weights of mice were significantly decreased by the administration of a CUR diet [40]. Orally ingested CUR also reversed the metabolic derangements associated with diabetes in mouse models of Type 2 diabetes [41]. CUR is known to be poorly absorbed from the gastrointestinal tract. It undergoes rapid metabolism and excretion, and thus has low bioavailability. However, with the co-administration of piperine, an active ingredient in black pepper, the systemic bioavailability of curcumin in rat and man could be increased by 2000% [42]. Taken together the micromolar concentration of curcumin employed in this study is conceivably attainable *in vivo*. The low toxicity of CUR, its excellent safety profile and the fact that it is a natural product consumed widely would make it an attractive candidate for treatment of obesity and diabetes. The control of obesity and diabetes through the modulation of AMPK has received considerable attention because the major metabolic response to exercise is mediated through AMPK [41].

Acknowledgments

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