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Green tea epigallocatechin gallate binds to and inhibits respiratory complexes in swelling but not normal rat hepatic mitochondria

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ABSTRACT

Epigallocatechin gallate (EGCG), the major flavonoid in green tea, is consumed via tea products and dietary supplements, and has been tested in clinical trials. However, EGCG can cause hepatotoxicity in humans and animals by unknown mechanisms. Here EGCG effects on rat liver mitochondria were examined. EGCG showed negligible effects on oxidative phosphorylation at 7.5–100 μM in normal mitochondria. However, respiratory chain complexes (RCCs) were profoundly inhibited by EGCG in mitochondria undergoing Ca^{2+} overload-induced mitochondrial permeability transition (MPT). As RCCs are located in mitochondrial inner membranes (IM) and matrix, it was reasoned that EGCG could not readily pass through IM to affect RCCs in normal mitochondria but may do so when IM integrity is compromised. This speculation was substantiated in three ways. (1) Purified EGCG-bound proteins were barely detectable in normal mitochondria and contained no RCCs as determined by Western blotting, but swelling mitochondria contained about 1.5-fold more EGCG-bound proteins which included four RCC subunits together with cyclophilin D that locates in mitochondrial matrix. (2) Swelling mitochondria consumed more EGCG than normal ones. (3) The MPT blocker cyclosporine A diminished the above-mentioned difference. Among four subunits of RCC II, only SDHA and SDHB which locate in mitochondrial matrix, but not SDHC or SDHD which insert into the IM, were found to be EGCG targets. Interestingly, EGCG promoted Ca^{2+} overload-induced MPT only when moderate MPT already commenced. This study identified hepatic RCCs as targets for EGCG in swelling but not normal mitochondria, suggesting EGCG may trigger hepatotoxicity by worsening pre-existing mitochondria abnormalities.

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1. Introduction

Epigallocatechin gallate (EGCG) is the abundant flavonoid in green tea (Camellia sinensis), tea products and dietary supplements (DS), all of which have a long, worldwide history of consumption. Numerous beneficial health effects are ascribed to EGCG [1], notably cancer chemoprevention, and despite numerous publications on this property, a review by the U.S. Food and Drug Administration (FDA) in 2011 concluded "there is no credible evidence to support" this claim. (http://www.fda.gov/Food/IngredientsPackagingLabeling/LabelingNutrition/ucm072774.htm). To date, only one product of EGCG-containing green tea extract (Brand name Veregen®) was approved by the FDA for topical use to treat external genital and perianal warts. Nevertheless, clinical trials are on-going to develop EGCG into a new oral anti-leukemia drug [2].

Despite the interest in potential health benefits, there have been considerable concerns over the hepatotoxicity associated with EGCG. For example, the DS Exolise, which contains a high level of EGCG, was withdrawn from the French and Spanish market in 2003 due to its possible association with liver toxicity [3]. Another EGCG-containing DS, Muscletech Hydroxycut, was cited by FDA for its liver risks and recalled from the U.S. market in 2009 [4]. In a recent phase 2 clinical trial, 6 out of 42 patients

Abbreviations: EGCG, epigallocatechin gallate; RCCs, respiratory chain complexes; MPT, mitochondrial permeability transition; IM, inner membranes; SDHA, succinate dehydrogenase complex, subunit A; SDHB, succinate dehydrogenase complex, subunit B; SDHC, succinate dehydrogenase complex, subunit C; SDHD, succinate dehydrogenase complex, subunit D; CsA, cyclosporine A; DS, dietary supplements; GTE, green tea extract; IC_{50}, half maximal inhibitory concentration; NDUFS3, NADH dehydrogenase [ubiquinone] iron-sulfur protein 3; NDUFS8, NADH dehydrogenase [ubiquinone] iron-sulfur protein 8; MGST1, microsomal glutathione S-transferase 1; ATP, adenosine triphosphate; MT-CO1, mitochondrially encoded cytochrome c oxidase I; UQCRC1, ubiquinol-cytochrome c reductase core protein I; UQCRC2, ubiquinol-cytochrome c reductase core protein II; HPLC, high-performance liquid chromatography; FDA, U.S. Food and Drug Administration.

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had to discontinue oral EGCG treatment due to significantly elevated transaminases [2], an indication of risk for severe liver injury [5]. High doses of EGCG have also been described as hepatotoxic in mice [6–8]. Yet several groups proposed that EGCG was protective against ethanol or carbon tetrachloride induced liver injury [9,10]. Recent work has shown that the timing of administration may be critical. Green tea extract (GTE), with a high proportion of EGCG, prophylactically prevented the onset of acetaminophen hepatotoxicity and unexpectedly enhanced such toxicity when GTE was administered shortly after the acetaminophen dose [11]. Thus, a clear mechanistic understanding of EGCG associated hepatotoxicity, directly or indirectly, is lacking.

Mitochondrial impairment has long been recognized as an important mechanism for drug or chemical induced hepatotoxicity [12]. Recent studies suggest that mitochondrial liability assessed *in vitro* is predictive of a compound's potential to induce low incidence human hepatotoxicity [13,14]. However, EGCG effects on hepatic mitochondria remain poorly understood. In isolated mouse hepatocytes, EGCG slightly decreased mitochondrial membrane potential at a very high concentration of 200 μM [8]. In rat liver submitochondrial particles, respiratory chain complex V (RCC V) was inhibited by EGCG with a half maximal inhibitory concentration (IC_{50}) of 17 μM [15]; however, EGCG effects on other RCCs (I–IV) have not been investigated, nor the mechanisms by which EGCG disrupts mitochondrial function. This study was undertaken to elucidate EGCG effects on isolated functional mitochondria, focusing on RCC activities. EGCG has been shown to covalently bind to cytochrome P450s in rat liver microsomes [16] and other proteins [17]; thus it was hypothesized that EGCG inhibits RCCs by covalently binding to their critical subunits.

2. Materials and methods

2.1. Chemicals, reagents and antibodies

EGCG with a purity of 98.5% was purchased from Blue California (Rancho Santa Margarita, CA, USA). The following antibodies were obtained from Abcam (Cambridge, MA, USA): (1) MitoProfile® Total OXPHOS Rodent WB Antibody Cocktail (catalog number: ab110413) which contains five antibodies. (2) MitoProfile® Membrane Integrity WB Antibody Cocktail (catalog number: ab110414) which also contains five antibodies. (3) RCC II WB Antibody Cocktail (catalog number: ab110410) which contains three antibodies. (4) Anti-succinate dehydrogenase complex subunit C (SDHC) (catalog number: ab155999) and (5) Anti-NADH dehydrogenase [ubiquinone] iron-sulfur protein 3 (NDUFS3) antibody (catalog number: ab110246). Anti-succinate dehydrogenase complex subunit D (SDHD) antibody was purchased from EMD Millipore Corporation (Darmstadt, Germany, catalog number: ABT110).

2.2. Animal care

Male Sprague–Dawley rats weighing 250–400 g were provided by the FDA's National Center for Toxicological Research (NCTR) breeding colonies. Animal care and experimental procedures were performed in accordance with the National Institutes of Health (NIH) "Guide for the Care and Use of Laboratory Animals" and were authorized by the NCTR Institutional Animal Care and Use Committee. Filtered tap water and NIH-41 irradiated diet were provided ad libitum.

2.3. Isolation of rat hepatic mitochondria

Rat liver mitochondria were prepared using a published method [18] with the following modification. Rats were anesthetized with pentobarbital and the liver was perfused using isolation buffer (70 mM sucrose, 190 mM mannitol, 20 mM HEPES, 0.2 mM EDTA, pH 7.5) to remove blood. The liver was homogenized using a 100 ml Dounce type homogenizer. Mitochondrial protein concentration was determined by the Bradford method [19].

2.4. Treatment of isolated mitochondria

Mitochondrial preparations (1 mg/ml, 20 ml), energized as described [18], were incubated with 0, 3.8, 7.5, 15, 30, 45, 60, 100 μM EGCG at room temperature for 30 min prior to downstream experiments. A small volume (2–3 ml) of the treated mitochondria were centrifuged and resuspended in 25 mM potassium phosphate pH 7.2 and subjected to three cycles of freezing-and-thawing (in preparation for measuring the activities of RCC I–V); the remaining samples were resuspended in 100 mM Tris–HCl buffer (pH 8.6) containing 0.5% Triton X-100 and 0.5% SDS to carry out the purification of EGCG-bound proteins. Where indicated, cyclosporine A (CsA) at 1 μM was added to mitochondrial preparations for 3 min before exposure to EGCG and/or Ca^{2+}.

2.5. Mitochondrial swelling assay

Mitochondrial swelling was measured using a Synergy 2 Multi-Mode Microplate Reader (BioTek, Winooski, VT, USA) by monitoring the decrease in absorbance at 535 nm every 2 min for a period of up to 30 min, as detailed in a previous publication [18].

2.6. Measurement of RCC activities

RCC I–V activities were measured using established protocols as detailed before [20], except that a smaller reaction volume of 0.2 ml was used.

2.7. Isolation of EGCG-bound proteins

Proteins with covalently bound EGCG were purified by the procedure of Weng et al. [16] with the following modifications: treated mitochondrial preparations (1 mg/ml, 13 ml per treatment group) were centrifuged at 7200*g* for 15 min. The supernatant was transferred to 15 ml tubes containing 13 mg ascorbic acid and 0.325 mg EDTA, and was immediately used to measure EGCG concentrations by HPLC. The mitochondrial pellets were re-suspended in 15 ml 100 mM Tris–HCl buffer (pH 8.6) containing 0.5% Triton X-100 and 0.5% SDS, and the suspension was shaken at room temperature for 1 h to solubilize proteins. Samples were centrifuged at 7200*g* for 20 min to remove any undissolved proteins and the supernatant was carefully transferred to new tubes containing 0.3 ml settled resin to start the affinity purification. Purified EGCG-binding proteins were eluted with 2 ml glycine (pH 2.0) buffer from the affinity resin and concentrated using a CentriVap® refrigerated centrifugal concentrator (Labconco Company, Kansas, Missouri, USA) to 0.2 ml after neutralization.

2.8. Western blotting

Proteins were mixed with 3 × reducing Laemmli buffer. Samples were not boiled. SDS–PAGE and Western blotting were carried out as previously described [16].

2.9. Analysis of free EGCG using HPLC

Free EGCG was extracted according to a published procedure [21]. The Dionex (Bannockburn, IL) Application Note 275 (http://www.dionex.com/en-us/webdocs/110783-AN275-HPLC-

Catechins-Tea-19May2011-LPN2799.pdf) was followed to measure EGCG concentration.

2.10. Statistic analysis

GraphPad Prism version 6.0 for Windows (GraphPad Software, Inc., CA, USA) was used to perform statistical analysis and generate figures. One-way analysis of variance (ANOVA) followed by Dunnett's post hoc test were used to compare among or between treatment groups. $p < 0.05$ was considered as statistically significant.

3. Results

3.1. EGCG showed no or minimal effects on oxidative phosphorylation in normal mitochondria

As a previous report showed EGCG suppressed RCC V in submitochondrial particles with an IC_{50} of 17 μM [15], it was anticipated there would be a change in oxygen consumption in normal mitochondria after ECGC treatment. However, EGCG at 3.75–100 μM showed no effects in either substrate-driven or ADP-driven respiration (data not shown). Then we sought to determine the activity of RCC V after EGCG treatment, and again there were no significant effects, though the activities of RCC I–IV were slightly decreased at 100 μM EGCG, with those of RCC II and III being statistically different from the control (Fig. 1).

3.2. EGCG inhibited RCC activity in swelling mitochondria

The failure to extend the EGCG effect reported in submitochondrial particles to normal mitochondria prompted the hypothesis that EGCG might not readily pass through the mitochondrial inner membrane (IM) or get into the matrix and therefore would be excluded from interacting with the RCCs located in these mitochondrial compartments. To test this hypothesis under pathophysiologically relevant situations, mitochondria undergoing Ca^{2+} (50 μM) overload-induced permeability transition (MPT) were used (Preliminary experiments confirmed the occurrence of MPT and relevant data were presented under Section 3.6.). As shown in Fig. 1, activities of RCC I–V were all significantly inhibited by EGCG in swelling mitochondria, with RCC I and II being suppressed even at 15 μM, a concentration close to that observed in the blood of some individual human subjects [22]. When swelling was blocked by CsA, which had no effects on RCC activities *per se*, EGCG inhibition of RCCs was nearly completely abolished (Fig. 1).

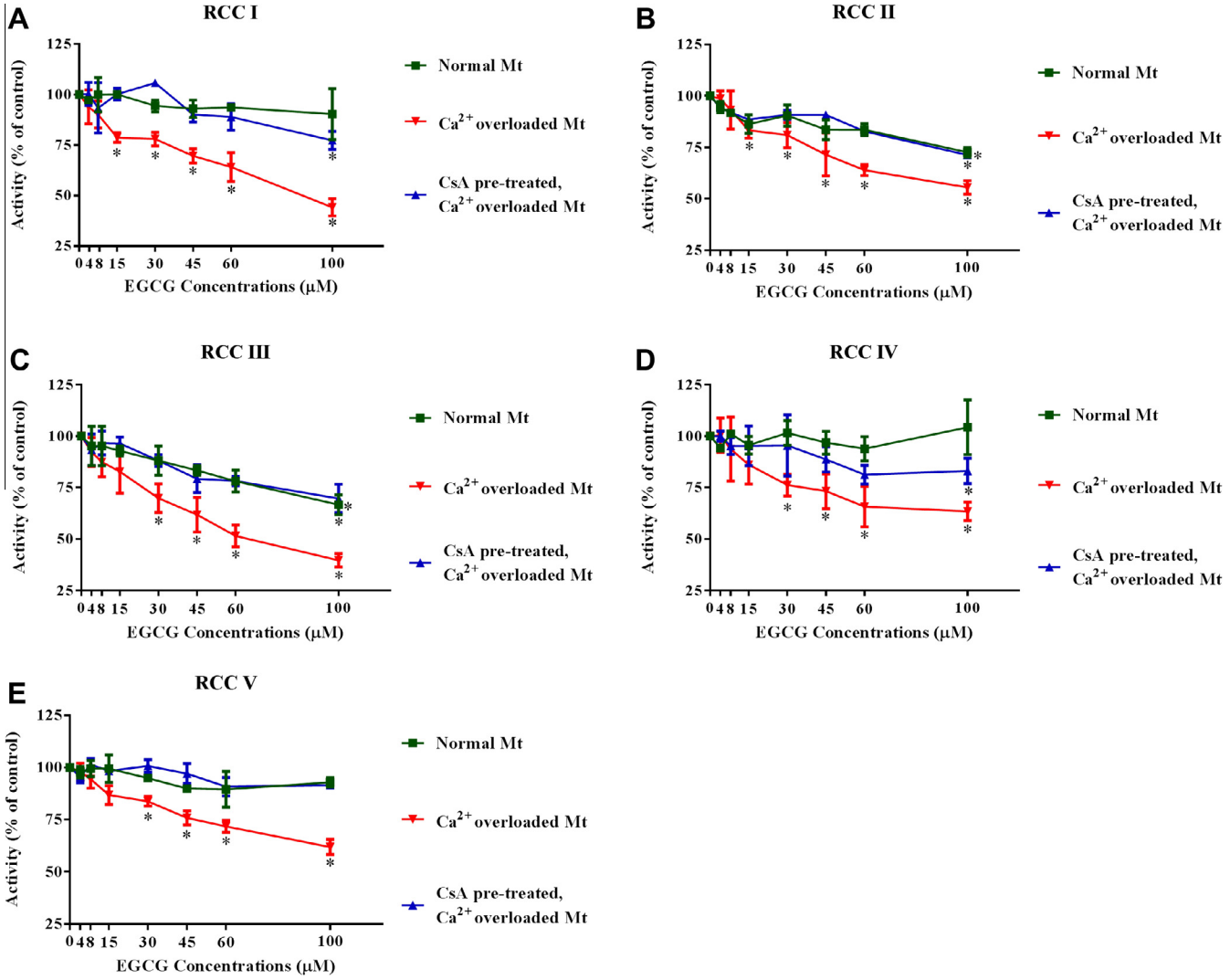

Fig. 1. Effects of EGCG on activities of RCC I (A), II (B), III (C), IV (D), and V (E) in rat liver mitochondria under different conditions. Normal mitochondria (Mt), Ca^{2+} (50 μM) overloaded swelling Mt, and CsA (1 μM)-pretreated, Ca^{2+} overloaded Mt were incubated with 0–100 μM EGCG for 30 min, and then RCC activity were measured. Data are mean ± SD, $n = 3$. The activity of RCC I–V in the control group was 30.7 ± 0.7, 120.0 ± 7.2, 287.8 ± 13.7, 471.4 ± 43.9, and 376.5 ± 4.1 nmol/min/mg protein, respectively. *$p < 0.05$, compared to the corresponding control groups that were treated with 0 μM EGCG.

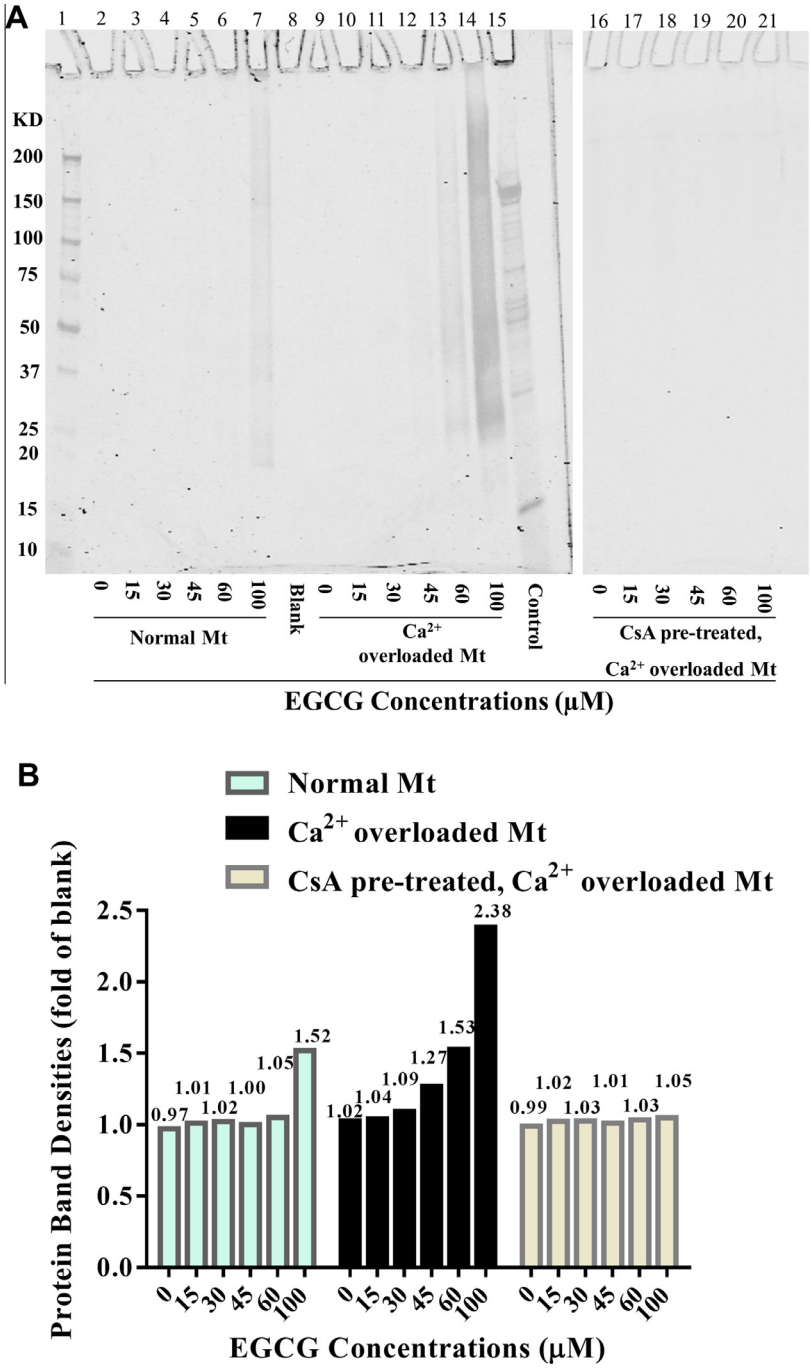

Fig. 2. Silver staining (A) and quantification (B) of isolated EGCG-bound proteins after SDS–PAGE. Mitochondria (Mt) under different states were incubated with EGCG for 30 min, and then EGCG-bound proteins were purified, followed by SDS–PAGE. Lane 1 contained protein markers and Lane 15 contained unfractionated mitochondrial proteins (5 μg) serving as a positive control. Lane 8 was blank loaded with the 1 × Laemmli buffer only. The protein band density on the gel (A) was quantified using the Image Studio Software (LI-COR Biosciences, Lincoln NE), normalized to the blank (Lane 8 in A), and presented in (B). Data are from three separate experiments.

To further test the hypothesis regarding EGCG effects depend on IM permeability, EGCG-bound proteins were isolated and detected by silver staining (Fig. 2) and Western blotting (Fig. 3).

3.3. EGCG bound to more proteins in swelling mitochondria than normal mitochondria

In Fig. 2A, no protein bands were observed in 0–60 μM EGCG treated normal mitochondria (lanes 2–6), and only very faint signals were seen in 100 μM EGCG treated samples (lane 7). However, in swelling mitochondria, multiple proteins bands were observed in 60 and 100 μM EGCG treated samples (lanes 13–14). In mitochondria pre-treated with CsA to prevent swelling, no protein bands were detected across all treatment groups (lanes 16–21). The "smearing" of protein bands were consistently observed in EGCG-bound proteins, likely due to protein cross-linking [16]. To quantify the result, protein band densities were obtained using the Image Studio Software and normalized to those of lane 8 (blank, Fig. 2A), in which only the 1 × Laemmli buffer was loaded. As shown in Fig. 2B, 60–100 μM EGCG generated about 1.5-fold more EGCG-bound proteins in swelling mitochondria than in normal mitochondria, and CsA pre-treatment decreased the EGCG-bound protein to control level.

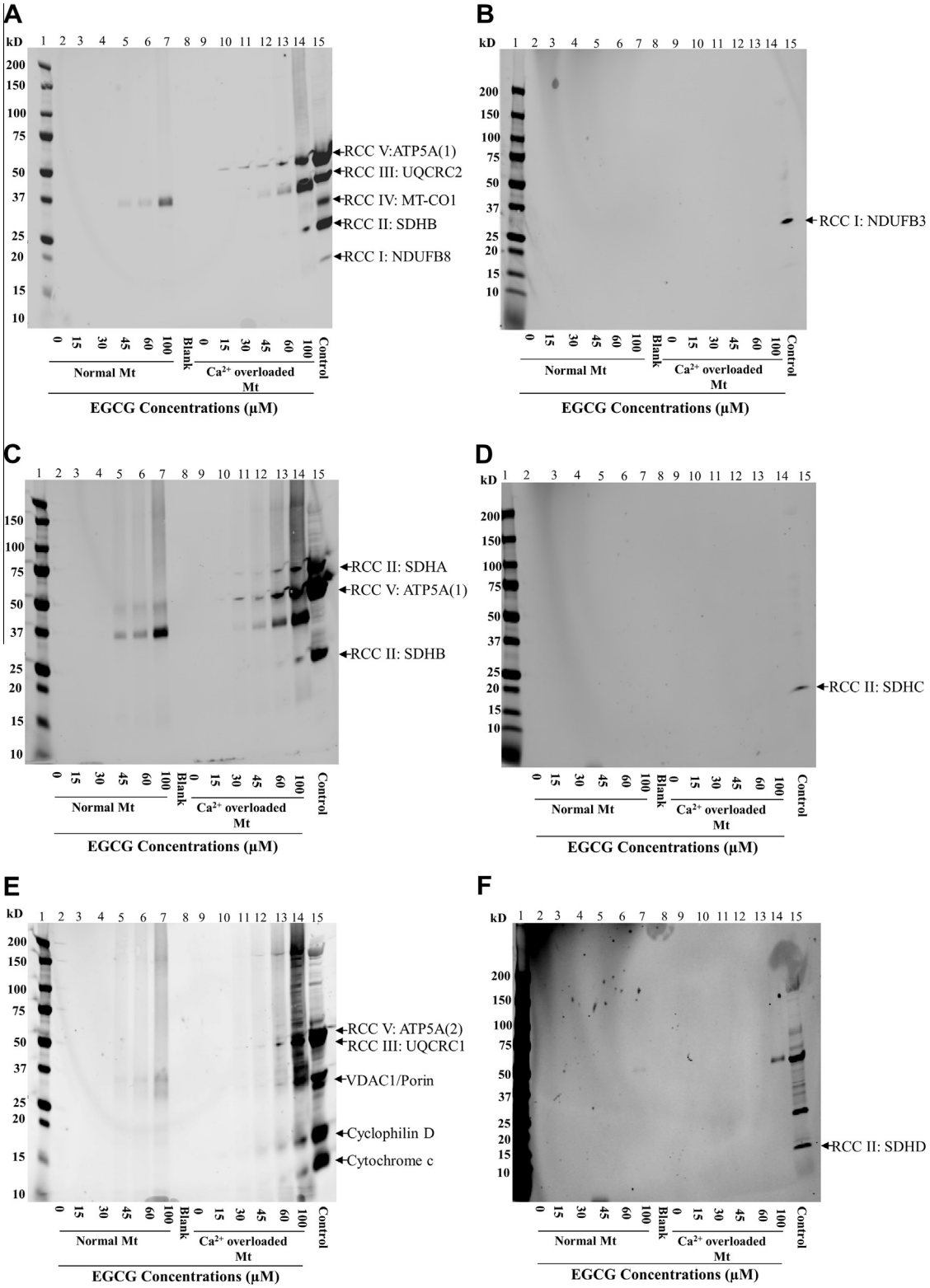

Fig. 3. EGCG-bound proteins detected by Western blot. Mitochondria (Mt) under different states were incubated with EGCG for 30 min. EGCG-bound proteins were purified and probed with MitoProfile® Total OXPHOS Rodent Antibody Cocktail (Fig. 3A), Anti-NDUFB3 antibody (Fig. 3B), RCC II Antibody Cocktail (Fig. 3C), anti-SDHC antibody (Fig. 3D), MitoProfile® Membrane Integrity Antibody Cocktail (Fig. 3E), and anti-SDHD antibody, respectively. Data are representative of at least three separate experiments.

3.4. EGCG selectively bound to certain RCC subunits

In Fig. 3A and B, no signals were obtained from anti-NDUFB8 or anti-NDUFB3, both of which are anti-RCC I antibodies, among the isolated EGCG-bound protein (lanes 2–7 and 9–14 in Fig 3A and B, respectively), though positive signals were seen in unfractionated mitochondrial proteins (lane 15 in the same figures).

As for RCC II, Fig. 3C shows that SDHA (lanes 13–14) and SDHB (Fig. 3A and C, lane 14) were positively detected among EGCG-bound proteins isolated from 60 to 100 μM EGCG treated swelling

Table 1
Free EGCG concentrations in the mitochondrial suspension.

EGCG added to mitochondria (μM)	EGCG (μM) remaining after reacting with mitochondria		
	Normal mitochondria	Ca^{2+} overloaded swelling mitochondria	CsA pre-treated, Ca^{2+} overloaded mitochondria
7.5	2.66 ± 0.07	1.56 ± 0.18*	2.37 ± 0.24
15	5.50 ± 0.16	3.56 ± 0.51*	5.16 ± 0.20
30	12.96 ± 2.02	9.28 ± 0.90*	12.16 ± 1.51
45	20.79 ± 2.20	15.87 ± 0.36*	19.76 ± 2.20
60	27.13 ± 3.12	21.40 ± 1.55*	26.65 ± 2.20
100	38.84 ± 3.29	37.09 ± 3.27	46.96 ± 2.82*

EGCG (7.5–100 μM) was incubated with mitochondria under different conditions for 30 min. Mitochondria were centrifuged to remove the protein and the supernatant was used to extract free EGCG, which was then measured by HPLC. Data are mean ± SD, $n = 3$.
* $p < 0.05$ when compared to normal mitochondria.

mitochondria but not among those isolated from normal mitochondria. SDHC and SDHD were undetectable in EGCG-bound proteins (Fig. 3D and F, lanes 2–7 and 9–14, respectively), though both were readily detectable in unfractionated mitochondrial proteins (lane 15 in corresponding figures).

Regarding RCC III, Fig. 3E shows that UQCRC1 was positively detected among EGCG-bound proteins of 45–100 μM EGCG treated swelling mitochondria (lanes 12–14) but not those of normal mitochondria (lanes 2–7), and Fig. 3A shows that UQCRC2 was not detectable in EGCG-bound proteins, though some cross-reacting bands were found with a slightly smaller molecular weight.

Anti-MT-CO1 contained in the MitoProfile cocktail was used to detect RCC IV. Fig. 3A shows that MT-CO1 was not detectable among EGCG-bound proteins (lanes 2–7 and 9–14), though it was positively detected in unfractionated mitochondrial proteins (lane 15).

Two different antibodies against the same subunit alpha subunit of RCC V (anti-ATP5A) were used for the detection of RCC V. As shown in Fig. 3A, the first anti-ATP5A antibody, which is designated as ATP5A(1) (Abcam category number: ab14748), gave positive signals in EGCG-bound proteins from 7.5 to 100 μM EGCG treated swelling mitochondria but not those from normal mitochondria, though the signal at 7.5 μM EGCG could not always (1 out 5 independent purifications) be detected (Fig. 3C). Fig. 3E shows that the second anti-ATP5A antibody, which is designated as ATP5A(2) (Abcam category number:ab110273), gave positive signals only in EGCG-bound proteins from 60 to 100 μM EGCG treated swelling mitochondria but not those from normal mitochondria. Fig. 3E also shows that cytochrome c and cyclophilin D were positively detected among EGCG-bound proteins from 100 μM EGCG treated swelling mitochondria but not those from normal mitochondria. The outer membrane protein isoforms of VDAC1/Porin were found to bind to EGCG in both normal and swelling mitochondria treated with 60–100 μM EGCG.

All EGCG-bound proteins detectable using Western blotting as shown in Fig. 3 were abolished when mitochondria were treated with 1 μM CsA prior to EGCG exposure (data not shown).

3.5. Swelling mitochondria consumed more EGCG than normal mitochondria

The next experiment determined free EGCG concentrations in the supernatant of different mitochondrial preparations. For 7.5–60 μM EGCG treated samples, the free EGCG concentration was much higher in the supernatant of normal mitochondria than

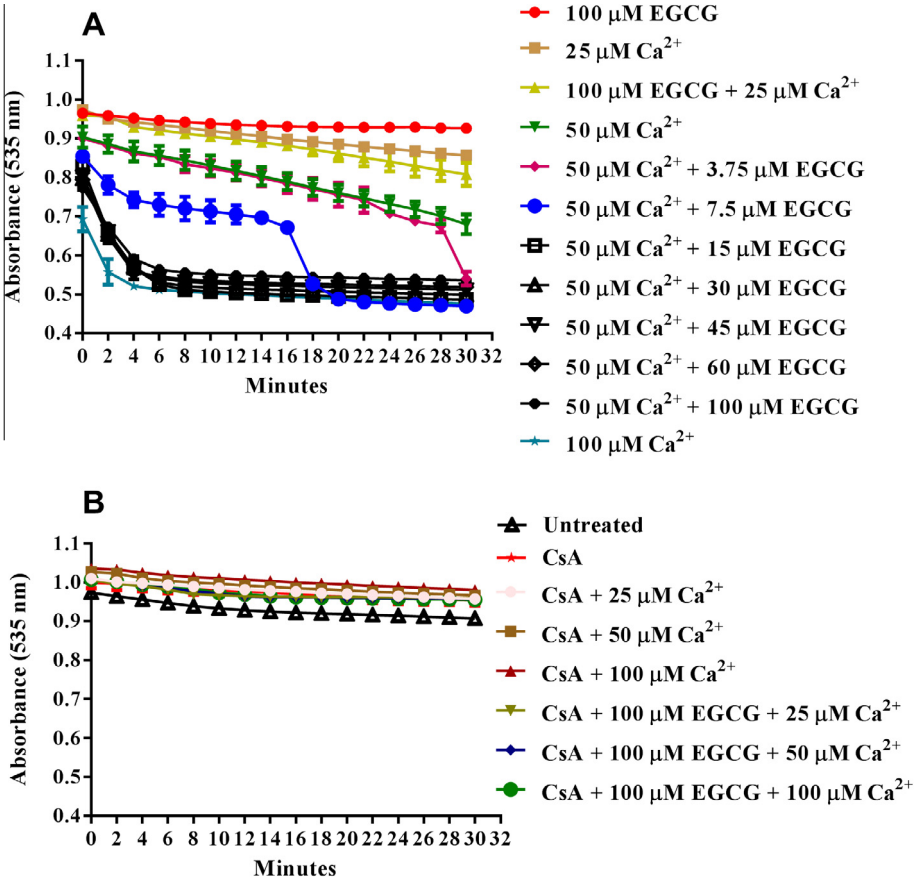

Fig. 4. Effects of EGCG on mitochondrial swelling induced by Ca^{2+} overload. Rat liver mitochondria were energized and pre-treated with (B) or without (A) CsA (1 μM), and then treated with Ca^{2+} and/or EGCG. The absorbance of 535 nm was measured for 30 min to determine mitochondrial swelling. Data are mean ± SD, $n = 3$.

swelling mitochondria, but was comparable to CsA pretreated, Ca^{2+} overloaded mitochondria (Table 1). In contrast, among 100 μM EGCG treated samples, a comparable free EGCG level was observed between the supernatant from normal and swelling mitochondria, but a much higher level was obtained in CsA pretreated, Ca^{2+} overloaded mitochondria (Table 1).

3.6. EGCG promoted MPT only when moderate MPT had occurred

EGCG effects on Ca^{2+} overload induced MPT was measured by mitochondrial swelling assay [18]. As shown in Fig. 4, EGCG at even 100 μM did not affect MPT in the absence of Ca^{2+}. In the presence of 25 μM Ca^{2+}, negligible MPT occurred, which was slightly enhanced by 100 μM EGCG. When 50 μM Ca^{2+} was used, moderate MPT occurred over time, and this concentration of Ca^{2+} was chosen in assessing EGCG effects on RCC activities. Interestingly, it was found that 50 μM Ca^{2+} induced MPT was remarkably enhanced by even 3.8 μM EGCG, a clinically-relevant concentration [22]. The swelling was completely prevented by CsA. The untreated mitochondria also underwent very sight swelling which was CsA-preventable as well. At the end of swelling assay, Western blot detection of cytochrome c release confirmed that the mitochondrial outer membrane integrity was not compromised (data not shown).

4. Discussions

The most significant finding of this study is that EGCG exerted differential effects on normal and swelling mitochondria, implying that if MPT occurs prior to EGCG exposure, EGCG would not only enhance such dysfunction but also trigger additional detrimental effects such as inhibiting oxidative phosphorylation. As a checkpoint of cell death, when MPT occurs, cells would either die from apoptosis if adenosine triphosphate (ATP) levels are sufficient or from necrosis if ATP is too low [23–25]. It is conceivable cells undergoing mild MPT would likely switch cell death from apoptosis to necrosis if exposed to EGCG, due to EGCG inhibition of RCCs and the resultant ATP shortage. A wide array of diseases may be associated with dysfunctional MPT [26], and numerous drugs can cause or modulate MPT [13,14,27]. Our data provide a novel mechanism by which EGCG may interact with drugs or diseases that are associated with mitochondrial abnormalities.

EGCG's differential effects on normal and swelling mitochondria are very likely related to the permeability of the IM. Under normal conditions, the IM is impermeable to most molecules including protons [25]; EGCG could not readily pass through the IM. However, during MPT, the IM is permeable to molecules of <1500 Da [25], and thus EGCG with a molecular weight of 458 would easily cross the IM and enter the matrix. The consumption of EGCG (Table 1) and binding of EGCG to IM proteins (Figs. 2 and 3) provides strong evidence supporting this hypothesis.

This study builds on a previous report that only showed EGCG inhibition of RCC V. Here we demonstrated that all five RCCs were susceptible to EGCG inhibition, and provided mechanistic insights into this effect, namely, that covalent-binding of EGCG to subunits of RCC I, II and V might underlie the inhibition. The failure in detecting EGCG-binding to RCC III or IV is likely due to limited availability of antibodies to their various subunits. Mechanisms other than covalent binding might also apply, and indeed may be operative for inhibition of the RCCs. Of note, though protein cross-linking is involved in EGCG induced functional changes [28,16], we did not observe such changes in all the RCCs examined (data not shown).

The finding that EGCG bound to mitochondrial matrix protein cyclophilin D, possibly via cysteine residue(s) [17], is reminiscent of the previous report which showed that cysteine 203 of cyclophilin D was susceptible to S-nitrosylation which in turn inhibited its activation and prevented MPT [29]. On the other hand, cytochrome c lacks free cysteines and therefore its binding to EGCG must be thiol-independent, as suggested for its binding to microsomal glutathione S-transferase 1 (MGST1) [16]. The functional consequence of such binding awaits further investigation.

Mitochondria treated with 100 μM EGCG appeared to consume EGCG in a manner different from that of other groups (Table 1). This could be partially explained by a minimal level of spontaneous MPT that was blocked by CsA, thus preventing 100 μM EGCG from entering the IM and matrix. This is in line with the silver staining result in Fig. 2, which showed that CsA pre-treatment abolished the EGCG-bound protein in 100 μM EGCG treated samples. Another factor is that EGCG at 100 μM bound to normal mitochondria (Fig. 2) causes RCC inhibition (Fig. 1). This and other unknown changes may lead to aberrant EGCG consumption.

To conclude, this study demonstrates that several subunits of rat hepatic RCCs are novel targets for EGCG-binding and inhibition in swelling, but not normal, mitochondria. These findings indicate that EGCG may trigger hepatotoxicity by worsening pre-existing mitochondria abnormalities.

5. Disclaimer

This article is not an official guidance or policy statement of the U.S. Food and Drug Administration (FDA). No official support or endorsement by the FDA is intended or should be inferred.

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