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Quercetin can act either as an inhibitor or an inducer of the mitochondrial permeability transition pore: A demonstration of the ambivalent redox character of polyphenols

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ABSTRACT

The Ca^{2+}- and oxidative stress-induced mitochondrial permeability transition (MPT) plays an important role in phenomena ranging from tissue damage upon infarction to muscle wasting in some forms of dystrophy. The process is due to the activation of a large pore in the inner mitochondrial membrane. Anti-oxidants are considered a preventive and remedial tool, and mitochondria-targeted redox-active compounds have been developed. Plant polyphenols are generally considered as anti-oxidants, and thus candidates to the role of mitochondria-protecting agents. In patch-clamp experiments, easily oxidizable polyphenols induced closure of the MPT channel. In swelling experiments with suspensions of mitochondria, high (20–50 μM) concentrations of quercetin, the most efficient inhibitor, promoted instead the onset of the MPT. Chelators of $Fe^{2+/3+}$ and $Cu^{+/2+}$ ions counteracted this effect. Fluorescent indicators of superoxide production confirmed that quercetin potentiates $O_2^{\cdot-}$ generation by isolated mitochondria and cultured cells. Since this was not affected by chelating Fe and Cu ions, the MPT-inducing effect can be ascribed to a "secondary", metal ion-catalyzed production of ROS. These results are a direct demonstration of the ambivalent redox character of polyphenols. Their mode of action *in vivo* cannot be taken for granted, but needs to be experimentally verified.

1. Introduction

In mitochondria part of the electrons transported by the respiratory chain is delivered to oxygen in a process generating superoxide anion, $O_2^{\cdot-}$, and hence H_2O_2 and reactive oxy and peroxy radicals ([1] and Refs. therein). While this fraction may be as high as 2–3% *in vitro*, chronic production of reactive oxygen species (ROS) by mitochondria *in vivo* is probably much lower, difficult to estimate and variable [1]. Nonetheless, its relevance and potential impact are clearly demonstrated, among much other evidence, by the phenotype of transgenic mice or cells depleted of or sovraexpressing Mn-SOD [2,3].

Production of ROS by mitochondria is relevant under pathological circumstances, including cancer and ischemias. Besides being a factor in carcinogenesis [4] mitochondrial ROS have been linked to metastatic aggressiveness and angiogenesis in the tumoral mass ([5]; but see [6]). These effects are mediated by the activation of enzymes such as metalloproteases [7] and transcription factors, most notably HIF [8–10]. The intermediacy of ROS in ischemia/reperfusion (I/R) damage is also well established [1,11,12]. The downstream effector in this case is, to a considerable extent, the mitochondrial permeability transition (MPT) [13–16].

The latter phenomenon (reviews: [17–21]) is at the focus of this paper. Briefly stated, it consists in the opening of an unspecific permeation pathway capable of admitting solutes up to 1.5 kDa, the mitochondrial permeability transition pore (MPTP), in the inner mitochondrial membrane (IMM). This causes depolarization, uncoupling, loss of metabolites and respiration factors such as NADH from the mitochondrial matrix, ATP depletion, and, if prolonged, leads to necrotic cell death [14,15]. In studies with isolated mitochondria the MPT is most often studied by following its propagation in the population of suspended organelles by monitoring light scattering. This is possible because opening of the MPTP induces colloidosmotic swelling. This method is not well suited to provide information on pore dynamics: swelling of a single mitochondrion takes place in the time range of 1 s [22] and once the mitochondrion has swollen, it does not report on the state of the MPTP. This latter type of information can instead be obtained by patch-clamping the membrane of mitoplasts. This approach has allowed a detailed characterization of a high-conductance pore whose pharmacology matches that of the MPTP [17,23,24].

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A number of conditions and chemicals are known to affect the MPT. Ca^{2+} is a key effector: *in vitro* the MPT is difficult to induce unless at least μM levels of this ion are present in the mitochondrial suspension. Activation by ROS has long been known and indeed it is considered by some to be an essential feature of the MPT [25–27]. Enhanced ROS production by Ca^{2+}-treated mitochondria has been observed (e.g. [28]). Believed to be caused by Ca^{2+}-induced alterations of membrane lipid–protein interactions, it is a possible mechanism for the transduction of Ca^{2+} binding into MPT opening [25,26,28–31]. The role of thiols in permeability transition onset has been carefully characterized by Bernardi's group [32–35]. At least two classes of dithiol sites can be recognized, differing in terms of accessibility to reagents and to mitochondrial pools of glutathione and pyridine nucleotides. Another mechanistic model, not necessarily alternative, envisions an important role of cardiolipin: its conversion to CL hydroperoxide might favour the formation of the MPTP by IMM carriers [36], and the detachment of cytochrome *c*, which can thus take part in apoptosis [37,38].

The MPT is not only a target for cell-, organ- and life-saving efforts (e.g. [20,39–42]); it can also represent a tool for the elimination of unwanted, i.e., cancerous, cells. Its induction by redox stress is one of the strategies considered in the emerging field of mitochondrial medicine [43,44]. Cancer cells live under intrinsic redox stress and may thus be more vulnerable than normal ones [45].

Manipulation of the redox state of mitochondria to either inhibit or enhance MPT occurrence thus appears to be a worthwhile approach to the prevention and/or treatment of major pathologies. Since cells maintain mM levels of "redox buffers" (e.g. glutathione) this perspective implies that a way is found to obtain sufficient concentrations of redox-active compounds in mitochondria. Hence the importance of developing mitochondrion-targeted redox-active constructs, which has stimulated ground-breaking research aiming at the delivery of ROS-abating compounds (reviews: [46–53]). Conjugation to membrane-permeant cations, the most widely used technique, results in accumulation according to Nernst's law. We have recently used conjugation to triphenylphosphonium to produce mitochondriotropic derivatives of quercetin [54], and resveratrol [55].

From the point of view of redox action polyphenols are ambivalent compounds [56,57]. The same chemical properties that confer them anti-oxidant character may lead, under different conditions, to a pro-oxidant effect [58,59]. Some polyphenols, including flavonols, inhibit peroxidases [60,61], and may alter the redox poise of cells by acting on enzymes of this class, including the mitochondrial thioredoxin–peroxiredoxin system and mitochondrial phospholipid hydroperoxide glutathione peroxidase. They may thus hinder or enhance the permeabilization of the IMM, a potentially useful outcome either way.

To gain clues as to how polyphenols might act on the MPTP, we have studied the effects of some representative members of the family on it, observing the activity of the pore at the single-channel level in patch-clamp experiments. We have then used the induction of swelling of isolated mitochondria to verify the effects of one of the compounds most efficacious in electrophysiological experiments, quercetin, on suspensions of isolated organelles. The two experimental approaches led to apparently contradictory results, underscoring the importance of carefully considering experimental conditions when evaluating the biomedical effects of these potentially extremely useful natural compounds.

2. Materials and methods

2.1. Materials

Daidzein was purchased from LKT Laboratories (St. Paul, MN, USA), galangin was from Indofine (Hillsborough, NJ, USA). All other reagents were from Sigma/Aldrich/Fluka/Riedel de Haen or from sources specified in the text.

2.2. Cells and mitochondria

Human Colon Tumor (HCT116) cells [62] (kindly provided by B. Vogelstein) were grown in Dulbecco's Modified Eagle Medium (DMEM) (GIBCO) plus 10 mM HEPES buffer, 10% (v/v) fetal calf serum (Invitrogen), 100 U/ml penicillin G (Sigma), 0.1 mg/ml streptomycin (Sigma), 2 mM glutamine (GIBCO) and 1% nonessential amino acids (100× solution; GIBCO), in a humidified atmosphere of 5% CO_2 at 37 °C. HCT116 mitochondria were isolated as described in [63]. Rat liver mitochondria were prepared by standard differential centrifugation procedures and obtained as a suspension in 0.25 M sucrose and 5 mM Hepes/K^+, pH 7.4.

2.3. Electrophysiology

Patch-clamp experiments on mitoplasts were carried out essentially as described by [64]. A few μl of mitochondrial suspension were added to 1 ml of standard electrophysiology medium (150 mM KCl, 0.5 mM $CaCl_2$, 1 mM Pi, 20 mM Hepes, pH 7.4) in the patch-clamp dish, and allowed to settle for approximately 10 min. The dish was then extensively perfused with the same medium to remove the objects not attached to the bottom. At variance from [64], mitoplasts were not produced by osmotic shock, but by "spontaneous" swelling in the presence of Ca^{2+} and Pi. Under these permeability transition-inducing conditions the mitochondria swell producing objects with a diameter of 1–3 μm on which seals were established under symmetrical ionic conditions. Pipette resistance was 4.6 ± 0.4 MΩ ($N = 49$). Connection to the Ag/AgCl ground electrode compartment was via a 1 M KCl agar bridge. For inhibition, a few μl of concentrated inhibitor solution were added, and the bath contents were thoroughly mixed by withdrawing and re-adding aliquots with a Gilson pipette. Seal configuration was mitochondrion-attached, as confirmed by the polarity of the voltage dependence of the 107 pS channel [65]. Voltage was controlled by an Axopatch 200 unit, and Axon pClamp software was used for voltage control and data analysis. The voltages reported in this paper are those applied to the patch-clamp pipette interior. Current (cations) flowing from the pipette to the ground electrode is considered as positive and plotted upwards. The voltage was either controlled manually or applied as trains of sequential square pulses to ±20 mV with alternating polarity and a 0.1-second "rest" period at 0 mV between pulses. All data were filtered at 10 kHz and recorded on tape using a VR-10B (Instrutech) adaptor, and recovered later for off-line analysis.

2.4. Mitochondrial swelling and respiration assays

Mitochondrial volume changes were followed as pseudo-absorbance decrease at 540 nm in a multi-sample Kontron Uvikon 922 UV/Vis spectrophotometer. The experiments were initiated by the dilution of the mitochondrial suspension into 250 mM sucrose, 10 mM Hepes/K^+, 5 mM succinate/K^+, 1.25 μM rotenone, and 1 mM P_i/K^+, pH 7.4 (suspension buffer), supplemented with the desired concentration of $CaCl_2$ and/or inhibitors. The temperature was 20 °C. Swelling traces shown in the same figure were recorded simultaneously. Respiration was monitored as [O_2] decrease in a stirred, closed, thermostatted chamber containing a Clark electrode.

2.5. Fluorescence assays

The formation of fluorescent, oxidized species from dihydroethidine (HE) (Invitrogen/Molecular Probes) was used to monitor the production of $O_2^{\cdot-}$ in RLM suspensions [66]. Stock solutions were prepared as detailed in [67] and used as described in [68]. RLM were incubated at 0.5 mg prot ml^{-1} in suspension buffer in a stirred quartz cuvette in a Shimadzu RL-5000 spectrofluorimeter for 2–4 min to check for signal stability. Quercetin was then added, followed by HE. Excitation was at 470 nm (3 nm slits) and emission was monitored at

585 nm (10 nm slits). $O_2^{\cdot-}$ generation in cells was detected using the mitochondriotropic probe MitoSOX Red® (Invitrogen/Molecular Probes) used as specified by the producer. Cells were sown on coverslips and cultured for three days. They were then placed in a holder, incubated for 10 min with 1 μM MitoSOX Red in HBSS (in mM units: NaCl 136.9, KCl 5.36, $CaCl_2$ 1.26, $MgSO_4$ 0.81, KH_2PO_4 0.44, Na_2HPO_4 0.34, and glucose 5.55, pH 7.4 (with NaOH)), then washed three times, covered with 1 ml HBSS, and placed on the microscope stage. Additions were performed by withdrawing 0.5 ml of incubation medium, adding the desired solute to this aliquot or to 0.5 ml of fresh medium with identical composition, mixing, and adding back the solution into the chamber at a peripheral point. Excitation was at 500–520 nm, and fluorescence was collected at λ>570 nm. Images were automatically acquired at 1 min intervals using an Olympus Biosystems apparatus comprising an Olympus IX71 microscope and MT20 light source, and processed with Cell^R© software. Images (Fig. 4) are presented using the same display parameters; fluorescence intensities can thus be compared.

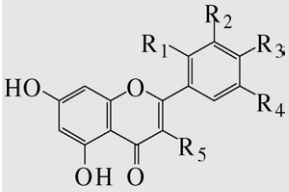
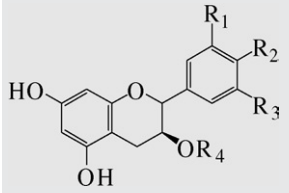
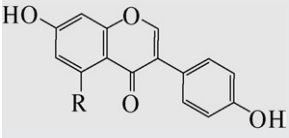
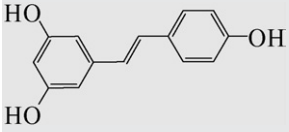
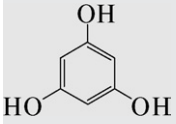
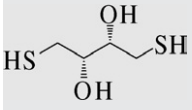
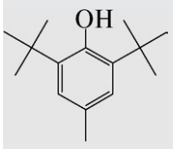
3. Results

3.1. Inhibition of the MPTP by readily oxidizable polyphenols

The most straightforward approach to an assessment of the effects of polyphenols on the MPTP is to observe the pore itself at the single-channel level in patch-clamp experiments, recording what happens when a polyphenol is added. We performed experiments of this type with a series of polyphenols (Table 1), using mitochondria isolated from rat liver (RLM) or from cultured HCT116 cells. Fig. 1 and Supplementary Figs. 1–3 illustrate representative experiments.

In these experiments, a computer-driven routine applied trains of 100 1-second, 20 mV "square" voltage pulses of alternating sign, separated by 100-ms "resting" zero-voltage intervals, to the mitoplast membrane patch. This protocol is routinely used in our lab in order to distinguish drug-induced from voltage-induced channel closure. A channel may stochastically close during a pulse, but it will re-open during the resting interval unless inhibited by the compound being

Table 1
Effects of selected polyphenols on MPTP activity in patch-clamp experiments.

Structure	Polyphenol	Concentration range (μM)	*N* Inhibition
	Myricetin R_1=H, R_2,R_3,R_4,R_5=OH	50	3 Yes(2)/no(1)
	Quercetin R_1,R_4=H, R_2,R_3,R_5=OH	5–50	27[a] Yes(24)/no(3)
	Morin R_2,R_4=H, R_1,R_3,R_5=OH	50	4 Yes(3)/no(1)
	Kaempferol R_1,R_2,R_4=H, R_3,R_5=OH	50	3 No
	Galangin R_1,R_2,R_3,R_4=H, R_5=OH	50	3 No
	Catechin R_1,R_4=H, R_2,R_3=OH	50	2 No
	Epigallocatechin (EGC) R_4=H, R_1,R_2,R_3=OH	50	2 No
	Genistein R=OH	50–100	2 No
	Daidzein R=H	50	5 No
	Resveratrol	50	2 No
	1,3,5-trihydroxybenzene	50	2 No
	Dithiothreitol (DTT)	2000	2 No
	Di-t-butyl-p-hydroxytoluene	200–500	5 Yes(4)/no(1)

[a] In the 3 negative experiments [quercetin] was 10 μM in 2 cases, 45 μM in the third.

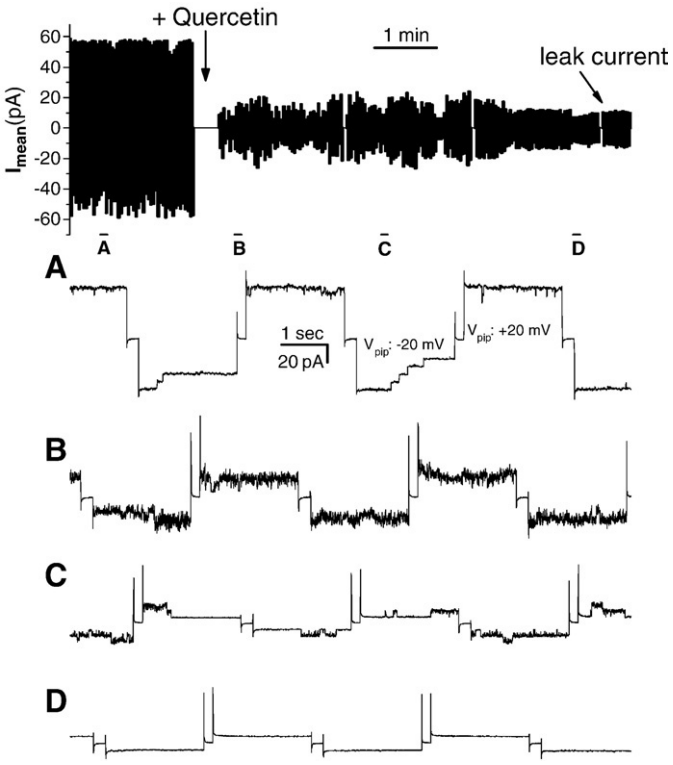


Fig. 1. Inhibition of the MPT pore by quercetin in patch-clamp experiments. In this representative experiment on an HCT116 mitoplast, 20-mV, 1-second voltage pulses of alternating polarity were applied sequentially, with a 0.1-second interval at zero voltage between pulses. Top panel: A column plot of the mean current flowing in the circuit during each 1-second pulse. Addition of 50 μM quercetin caused closure of the channels, i.e., a decrease of the mean current. (A–D) Current records corresponding as indicated to the plot segments identified by bars and letters in the top panel. Immediately after the addition of quercetin the patch still exhibited a residual activity characterized by a fast gating ("noisy" trace) which eventually disappeared leaving only the leak current (D). Sampling: 1 kHz. Filter: 200 Hz.

tested. The effect of the would-be inhibitor can then be appreciated at a glance by observing a graph plotting the mean current flowing in the circuit during each 1-second voltage pulse vs. time (top panel in Fig. 1). Channel activity was monitored for a control period, after which the desired compound was added. Since the experiments were conducted in the mitoplast-attached configuration, the effect, if any, took place after a variable lag time, inversely related to the concentration applied, needed for the diffusion from the bath to the membrane patch enclosed by the pipette rim. Activity was therefore generally monitored for at least two 100-pulse (110-second) trains after the addition before pronouncing the addition to have been ineffective.

The results are summarized in Table 1. For this tabulation, an experiment was arbitrarily considered to have produced inhibition if the open probability (calculated from the mean current, leak subtracted) of the megachannels in the patch was eventually reduced by at least 70%. According to this criterion, among the polyphenols tested quercetin (Fig. 1), myricetin and morin (Supplementary Fig. 1) were able to inhibit the MPTP in a majority of experiments. BHT, a widely used reducing agent, radical scavenger and MPTP inhibitor (e.g. [17] and Refs. therein) had an analogous effect (Supplementary Fig. 2). Catechin and epigallocatechin, which have hydroxylic substitution patterns in common with quercetin and myricetin, but are less readily oxidized, did not inhibit the pore (Table 1).

The current records reveal an interesting detail. In most experiments, the addition of an effective polyphenol initially caused only a partial inhibition of channel activity. The residual activity was characterized by a previously absent fast gating, which – especially if more than one active channel was present in the patch – generally appeared as irregular "open channel noise". This behaviour is clearly visible in Fig. 1, and particularly in Supplementary Fig. 1, an example chosen because morin induced a relatively slower and more regular gating pattern. The amplitude of this "flickering" generally decreased in time and in most experiments (quercetin: 19/24; myricetin: 1/3; morin: 1/3) a complete and permanent (i.e., lasting until the tight seal collapsed, at least a few minutes) disappearance of activity eventually set in within the timeframe of the experiment.

3.2. Quercetin induces the permeability transition in mitochondrial suspensions

While patch-clamp allows one to follow the behaviour of the individual channels "in real time", the most popular technique used to study the mitochondrial permeability transition relies on MPT-dependent colloidosmotic swelling of isolated mitochondria. We therefore investigated the effects of quercetin, the most reliable inhibitor in electrophysiological experiments, using that approach.

The addition of quercetin to mitochondria suspended in a standard sucrose-based buffer containing phosphate and a respiratory substrate but no added Ca^{2+} was without detectable effect (not shown). MPT induction generally requires Ca^{2+}, at concentrations depending on conditions and on the individual mitochondrial preparation and its "age". We checked therefore whether quercetin would inhibit MPT induction by Ca^{2+}. This was not the case. On the contrary, at concentrations >10 μM the polyphenol acted synergically to induce swelling (Fig. 2), which could be inhibited by CsA (Fig. 2B), an acidic (5.5) pH, ADP, Mg^{2+} and DTT, well known inhibitors of the MPT (Supplementary Fig. 4 and not shown). Respiration was analogously stimulated, and CsA inhibited this stimulation (not shown).

3.3. Quercetin induces production of $O_2^{\cdot-}$

Since ROS are MPT inducers, and polyphenols can function as pro-oxidants, we checked whether the presence of quercetin was associated with an increased formation of superoxide anion in our

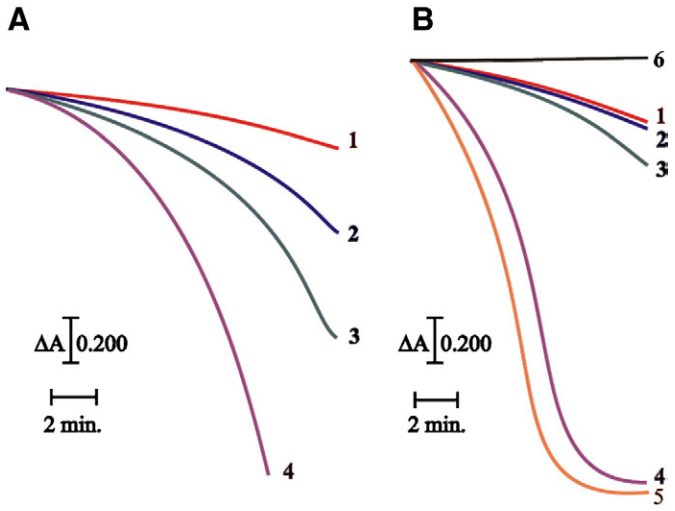


Fig. 2. Ca^{2+} and quercetin act synergically to induce swelling of mitochondria in suspension. Parallel light scattering experiments initiated by the addition of 1 mg prot ml^{-1} RLM to the cuvettes. (A) Increasing concentrations of quercetin (Q), at constant [Ca^{2+}], result in increasing rate of propagation of swelling in the mitochondrial suspension. The medium contained in all cases 40 μM $CaCl_2$ plus: trace 2: 25 μM Q; trace 3: 40 μM Q; trace 4: 50 μM Q. (B) Increasing concentrations of Ca^{2+}, at constant [quercetin], result in accelerated swelling. Cyclosporin A blocks swelling. The medium contained: trace 1: 40 μM $CaCl_2$; trace 2: 20 μM $CaCl_2$ + 40 μM Q; trace 3: 30 μM $CaCl_2$ + 40 μM Q; trace 4: 40 μM $CaCl_2$ + 40 μM Q; trace 5: 50 μM $CaCl_2$ + 40 μM Q; trace 6: 40 μM $CaCl_2$ + 40 μM Q + 1 μM Cyclosporin A. The traces shown are representative of 6 (A) and 5 (B) analogous experiments.

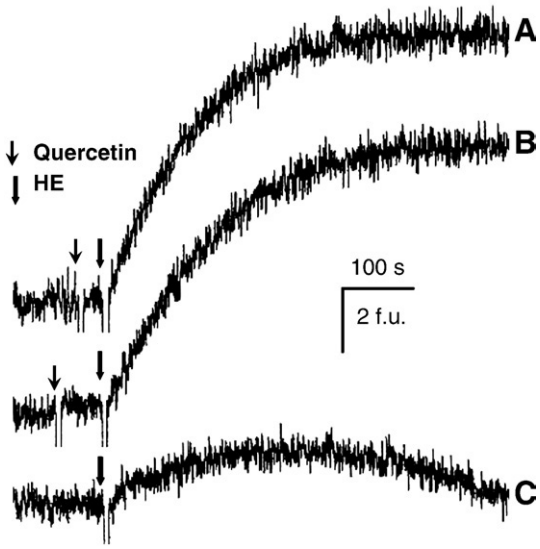


Fig. 3. Quercetin enhances superoxide generation by suspensions of mitochondria. 50 μM quercetin and 6.34 μM HE were added to the stirred suspension of RLM as indicated. Consecutive (A→C) recordings using the same preparation of RLM. For trace B the medium contained 10 μM bathophenanthroline and bathocuproine. For trace C (control), no quercetin was added. The traces shown are representative of 4 analogous experiments.

system. Oxidation of dihydroethidine (HE), a "leuco dye" specifically sensitive to $O_2^{\cdot-}$ [66–68], was indeed greatly enhanced by 50 μM quercetin (compare traces A and C in Fig. 3). Note also that the extent and kinetics of "ethidium" fluorescence development are the same regardless of whether the suspension medium contained (trace B) or not (trace A) specific iron and copper ion chelators (see below and Discussion). These experiments are complicated by the occurrence of the MPT: artefactual variations in the fluorescence signal are introduced by changes in light scattering due to swelling. To avoid this complication, observations of $O_2^{\cdot-}$ generation by the isolated RLM were carried out in the presence of CsA.

A very slow fluorescence increase took place in cultured HCT116 cells loaded with MitoSOX Red®, a hydroethidine derivative targeted to mitochondria by a phosphonium ion moiety [69,70], and exposed to quercetin (Fig. 4). Although slow, this increase was reproducible, and reproducibly absent in controls ($N=5$ and 3 respectively). As expected, it was markedly enhanced by the mitochondrial electron transport chain blocker Antimycin A, known as an inducer of superoxide production (e.g. [69]). These observations suggest a mild pro-oxidant effect of quercetin also within cells in culture.

3.4. MPT induction is due to Fe- and Cu-catalyzed ROS production

Superoxide anion is well known to be rapidly converted to H_2O_2 by SOD. Hydrogen peroxide can in turn give rise to very reactive hydroxy and peroxy radicals via metal-catalyzed Fenton-type reactions. Whether $O_2^{\cdot-}$ itself can act as an MPT inducer is unclear. A direct measurement of H_2O_2 production under our conditions was made difficult by the fact that flavonoids, including quercetin, inhibit the peroxidases used in most H_2O_2 assays (not shown; e.g.: [60,61,71]). A suspension of isolated mitochondria may be reasonably expected to contain reactive Fe and Cu species. We assessed the role of these metals by checking whether quercetin-enhanced swelling would be antagonized by specific chelators. Indeed bathophenanthrolin (BF;

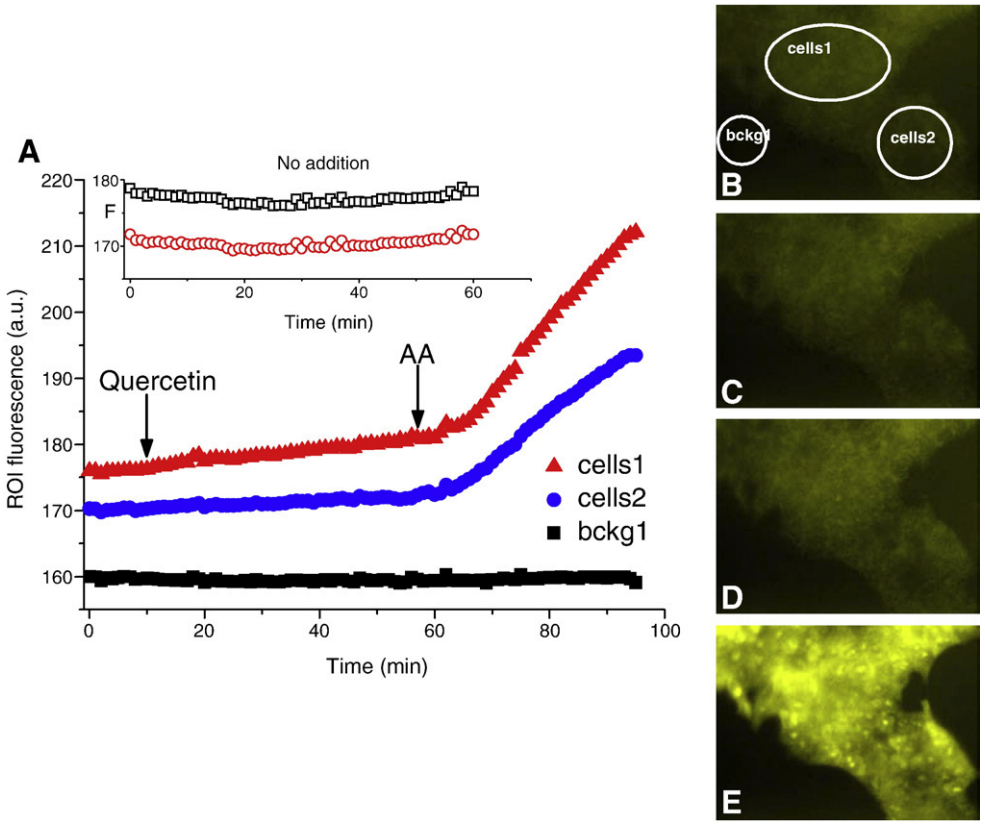


Fig. 4. Superoxide production in cells exposed to quercetin. A representative imaging experiment with MitoSOX Red®-loaded HCT116 cells. See Materials and methods for details. (A) A computer-generated plot of the fluorescence emitted by the field areas (Regions Of Interest, ROI) identified in panel B. Images were acquired every 60 s. The difference in the fluorescence of the ROIs "cells1" and "cells2" is due to the difference in the area covered. 50 μM quercetin and 2 μg/ml Antimycin A were added when indicated. The inset shows an analogous plot from two cell-filled ROIs in a control experiment in which quercetin was not added. (B–E: Representative images taken from the sequence used. (B) At time 0. (C) After 10 min, no addition. Quercetin was added seconds after this image was acquired. (D) After 46 min in the presence of 50 μM quercetin. Antimycin A was added seconds after this image was taken. (E) After 40 min in the presence of 2 μg/ml Antimycin A.

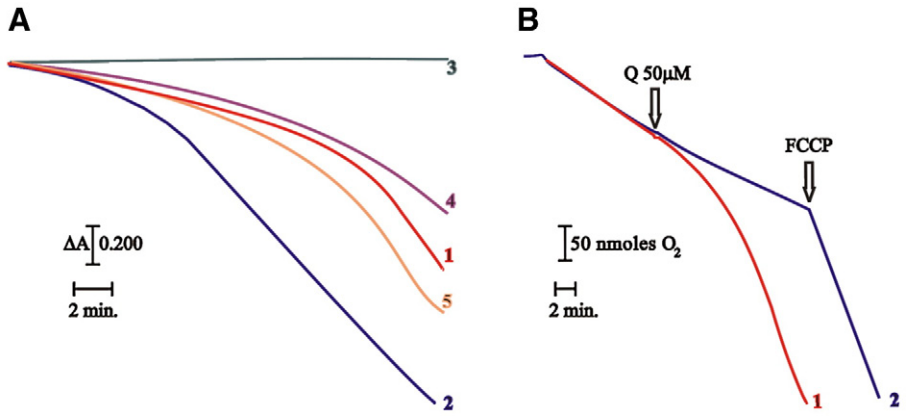


Fig. 5. Induction of the permeability transition by quercetin is mediated by Fe and Cu ions. (A) Swelling. All traces: the medium contained 50 μM $CaCl_2$. Further additions (from the beginning): trace 2: 50 μM Q; trace 3: 50 μM Q + 1 μM CSA; trace 4: 50 μM Q + 10 μM BF and BC; trace 5: 50 μM Q + 10 μM BF. (B) Respiration. When indicated, 50 μM Q was added in both runs, 1 μM FCCP only in 2. The medium of trial 2 contained 10 μM BF and BC. The data are representative of 6 analogous experiments.

4,7-diphenyl-1,10-phenanthroline; log K_{Fe}: 5.6; log K_{Cu}: 8.8) and bathocuproine (BC; 2,9-dimethyl-4,7-diphenyl-1,10-phenanthroline; log K_{Cu}: 19.1; log K_{Fe}: ~0), specific chelators of iron and copper, with negligible affinity for Ca^{2+}, strongly reduced swelling and the attendant respiration increase (Fig. 5A, B). Deferoxamine and penicillamine, two other iron and copper (respectively) chelating agents, had similar effects (not shown). As expected, the presence of added μM-range concentrations of Fe and Cu ions as well as of quercetin produced a rapid, Ca^{2+}-independent, CsA-insensitive pseudo-adsorbance decrease (not shown).

4. Discussion

The results show that readily oxidizable polyphenols, quercetin in particular, can both inhibit and activate the permeability transition pore, in a clear demonstration of the ambivalent nature of these redox-active compounds. Inhibition can be observed in patch-clamp experiments, conducted under well-controlled conditions, in the presence of very few mitoplasts (in a typical experiment only perhaps 100 remain in the 1-ml chamber after washing) bathed in a medium containing no transition metal ions. Activation is observed under different circumstances: a dense (typically 1 mg prot ml^{-1}) suspension of isolated organelles containing sequestrable iron and copper species capable of catalyzing quercetin oxidation with the production of $O_2^{\cdot-}$, H_2O_2 and other ROS which appear to be the proximal inducing agents.

A priori, inhibition might be due to molecular interactions, i.e., phenomena not involving a chemical reaction, or to redox events. What is known about the MPTP points to this latter hypothesis as the more likely of the two. Electrochemical data on polyphenols are scarce and vary depending on the experimental conditions used. Nonetheless, available $E_{p/2}$ values [72–74] are sufficient to indicate the following ease-of-oxidation ranking: myricetin, quercetin, morin>kaempferol, catechin>resveratrol>galangin>genistein, daidzein. These data correlate well with those in Table 1, and are thus consistent with a redox mechanism for MPT inhibition. Compounds with structure and functional groups similar to those of quercetin, e.g. kaempferol or catechin, but less readily oxidizable, do not readily inhibit the channel, whereas BHT, in which the bulky t-butyl groups presumably impede strong interactions of the lone hydroxyl group with proteins, does.

The fast gating induced in a first phase of the inhibition process may thus be associated with redox events, i.e., it may reflect the electron transfers and conformational changes involved in the transition between (the) oxidized specie(s) constituting the open state of the MPTP and the reduced and closed channel, and vice versa. These redox events are believed to be reversible thiol/disulfide interconversions (see Introduction). The current variations thus may represent a record of the forward and backward steps of a chemical reaction: reduction of a disulfide group would cause channel closure, its re-oxidation would precipitate opening. The frequency of these events would be expected to be a function of the concentration of the reagent at the site of action – which in turn depends on the geometry of the pipette/membrane system and on the time elapsed since the addition – and of its propensity to cede electrons. In some cases, such as the one in Supplementary Fig. 1, this would be low enough for the events to be well resolved. Alternatively, a reduction may alter the structure of the channel, destabilising both the open and the closed states (fast gating). In any case, a further, subsequent redox event would then block the protein in the closed-channel state (prolonged closure), explaining the eventual complete disappearance of any activity. The observed behaviour may thus be easily reconciled with the presence of multiple dithiol groups exerting control of MPTP activity [32–35].

It is relevant that in patch-clamp experiments quercetin nearly always induced channel inhibition. This implies that all the individual channels observed contained oxidized groups whose reduction caused channel disappearance, and thus that MPTP channel genesis always (or at least in the vast majority of cases) involves oxidation, presumably of thiols. Note that in the experiments reported here the mitochondria had been made to swell by incubation for a few minutes, in the patch-clamp dish, with Ca^{2+} and Pi, i.e., under classical MPT-inducing conditions, without any added oxidizing agents.

Presumably MPTP-inhibiting reduction of disulfide groups by quercetin also takes place in suspension experiments. This does not result in any detectable inhibition of the propagation of swelling in the population. This lack of effect is not surprising, since any anti-oxidant action is presumably overwhelmed by the generation of ROS. Furthermore, for the disulfide moieties to be reduced, they must first of all form via oxidation of thiols. Their formation is thought to coincide with PTP opening, which leads to complete swelling in a matter of hundreds of milliseconds [22]. It is also possible that the relevant reaction may be thiol arylation by products arising from oxidation of the quercetin catechol moiety, as proposed in the case of mangiferin, another polyphenol that can induce the MPT [75]. Subsequent closure of the pore is not expected to lead, except under specific experimental conditions [76] to shrinkage. Furthermore, more than one MPTP may be open in any given mitochondrion at any given time, and there is no reason to expect individual pores to synchronize their opening or closing. Just one open pore would be sufficient to induce swelling of its mitochondrion.

The protective effect of Fe/Cu chelation (Fig. 5) implies that "secondary", Fenton reaction-derived ROS, rather than $O_2^{\cdot-}$ itself, are mostly involved in the activation of the MPTP. This conclusion is based on the observation that BF and BC drastically slow down the

propagation of swelling but have little or no effect on the $O_2^{\cdot-}$ mediated formation of fluorescent HE-derived species in RLM suspensions (compare traces A and B in Fig. 3). Quercetin-promoted ROS production in cells (Fig. 4), although minor in comparison with a strong stimulus such as Antimycin, may well account at least in part for the antiproliferative/cytotoxic activity exhibited by readily oxidizable polyphenols at high concentrations [57,73,77,78].

Given that *in vitro* reactive polyphenols can act as either agonists or antagonists of the MPT depending on conditions as well as on their properties, it is difficult to predict how they would act *in vivo* [57]. Whether a flavonoid has a protective or cytotoxic action may well depend on its concentration [79]. This ambivalence is exemplified by the recent report that "mitoQ" [64], developed as an anti-oxidant agent, can also act as a pro-oxidant [80]. In electrophysiological experiments quercetin often succeeded in inhibiting the MPTP at low μM levels (see Supplementary Fig. 3). MPT-inducing effects have been observed only at higher (>10 μM) concentrations, in the presence of Ca^{2+}, and with dense suspensions of mitochondria. These conditions are not likely to occur *in vivo* upon consumption of quercetin-containing foods, since the bioavailability of polyphenols in foodstuffs is low and metabolism by enterocytes and hepatocytes is very effective [81–83]. On the other hand, other modes of administration may be envisioned in a therapeutic perspective, and pro-drugs are beginning to be considered as a strategy to enhance adsorption and to increase the levels of active species where needed (e.g.: [84]). Mitochondrial targeting by linkage of a triphenylphosphonium moiety is intended precisely to increase the concentration of the compound in mitochondria.

A variety of cancers endure oxidative stress and elevated levels of copper ions [85]. Our results suggest that these conditions may make them vulnerable to polyphenol-induced, MPT-mediated cell death if sufficient levels of oxidizable molecules are delivered. An anti-oxidant overall effect may also have oncological applications [5,7]. The results reported in this paper thus support a polyphenol-based approach to mitochondrial medicine, and stress the need for observations at the level of cellular and animal experimental systems.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbabio.2009.06.002.

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Glossary

ANT: adenine nucleotide translocator
BC: bathocuproine
BF: bathophenanthroline
BHT: 2,5-di-t-butyl-p-hydroxytoluene
CL: cardiolipin
CsA: cyclosporine A
FCCP: carbonyl cyanide p-trifluorometoxyphenyl hydrazone
HIF: hypoxia-inducible transcription factor
IMM: inner mitochondrial membrane
HE: dihydroethidine
HBSS: Hank's balanced salt solution
I/R: ischemia/reperfusio
MPTP: mitochondrial permeability transition pore
PiC: phosphate carrier
RLM: rat liver mitochondria
ROI: region of interest
ROS: reactive oxygen species
SOD: superoxide dismutase
VDAC: voltage-dependent anion channel (mitochondrial porin)