The dietary flavonoid quercetin activates BKCa currents in coronary arteries via production of H$_2$O$_2$. Role in vasodilatation

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Abstract

Objective: Large conductance Ca$^{2+}$-activated K$^+$ channels (BKCa) regulate coronary artery tone in vivo, play a key role in blood pressure regulation, and have been suggested as novel potential drug targets in hypertension. Quercetin exerts systemic and coronary vasodilator effects in vitro and reduces blood pressure in several rat models of hypertension, and its consumption is associated with a lower mortality rate from coronary heart disease in epidemiological studies. We hypothesized that quercetin might activate BKCa channel in isolated myocytes from rat coronary arteries and that this mechanism might be involved in its coronary artery relaxant effects.

Methods: Membrane currents were measured using the whole-cell configuration of the patch-clamp technique. Contractile tension was recorded in rat coronary artery rings mounted in a myograph.

Results: Quercetin ($\approx 0.1$ μM) increased the outward currents in the whole range of test potentials, hyperpolarized cell membranes, and increased the frequency of spontaneous transient outward currents (STOCs) carried by BKCa channels. These effects were abolished by the selective BKCa blocker iberiotoxin and by catalase. Quercetin increased dichlorofluorescein fluorescence in coronary arteries in a polyethylenglycol-catalase-sensitive manner, indicating that it increased cytosolic H$_2$O$_2$. The membrane-permeable analogue of H$_2$O$_2$ t-butylhydroperoxide mimicked the effects of quercetin on outward currents. The vasodilator effect of quercetin in isolated rat coronary arteries was partially inhibited by iberiotoxin.

Conclusion: Quercetin increased BKCa currents via production of intracellular H$_2$O$_2$. This effect is involved, at least partly, in the coronary vasodilator effects of quercetin.

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1. Introduction

K$^+$ channels play a key role in regulating resting arterial membrane potential and tone [1]. Activation of K$^+$ channels in vascular smooth muscle leads to hyperpolarization, decreased activity of voltage-gated L-type Ca$^{2+}$ channels, reduced [Ca$^{2+}$], and vasodilation. Large-conductance Ca$^{2+}$-activated K$^+$ channels (BKCa), formed by an ion-conducting $\alpha$ subunit and a regulatory $\beta_1$ subunit, are activated by depolarization and by increases in [Ca$^{2+}$], [2]. Local increases in [Ca$^{2+}$], near the plasma membrane (Ca$^{2+}$ sparks), caused by the release of Ca$^{2+}$ from the sarcoplasmic reticulum, are functionally coupled to BKCa channels whose activation produces spontaneous transient outward currents (STOCs), hyperpolarization and vasodilation [3,4]. BKCa channels are targets for vasoactive factors such as NO and endothelium-dependent hyperpolarizing factor (EDHF) [5,6]. Recent studies also suggest that BKCa channels may play a key role in blood pressure regulation. In fact, mice deficient in $\beta_1$ gene (kcnmb1) showed elevated blood pressure [7]. Furthermore, $\beta_1$ subunit downregulation has been reported in hypertensive rat models [8] and mutations
of the β1 subunit have been associated with a lower prevalence of diastolic hypertension and cardiovascular risk [9,10]. In addition, coronary artery tone is strongly dependent on the activity of BKCa channels [11]. Aging is associated with reduced expression and density of BKCa channels in coronary arteries from rats and humans [12,13] which is consistent with a higher frequency of spontaneous contractile activity and the increased risk of coronary vasospasm in older people [14]. Furthermore, the activity of BKCa channels is reduced in coronary arteries during left ventricular hypertrophy which could contribute to the reduced coronary reserve observed in such cases [15]. Therefore, BKCa channels have been suggested as novel potential drug targets for the treatment of cardiovascular diseases. Unfortunately, the known activators of BKCa channels are not very selective [16].

Flavonoids belong to a large group of plant polyphenols that are consumed in large amounts with dietary fruit and vegetables. The average daily flavonoid intake in the occidental diet is ≈ 23 mg, of which quercetin represents 60% to 75% [17]. Large epidemiological studies have shown an inverse association between dietary flavonoid intake and mortality from coronary heart disease [17,18]. Although prospective randomized clinical trials are lacking, several studies using animal models support these potential protective effects of flavonoids in cardiovascular diseases [19]. Quercetin exerts systemic and coronary vasodilator effects in vitro [20–24] and reduces blood pressure, cardiac hypertrophy, endothelial dysfunction, vascular remodelling and oxidative status in several rat models of hypertension [25,26]. However, quercetin may also behave as a pro-oxidant and generate hydrogen peroxide (H2O2) [27,28]. The mechanisms involved in the vasodilator effects of flavonoids are not fully understood but may involve inhibition of protein kinases such as protein kinase C and myosin light chain kinase [20,21]. Endothelium-dependent relaxation has been reported for several flavonoids; however, most studies with quercetin indicate that endothelium removal does not significantly change its vasodilator effect [20–24]. In addition, the vasodilator effects of some flavonoids were inhibited by the non-specific K+ channel blocker tetraethylammonium and the specific BKCa blocker iberiotoxin in rat mesenteric arteries [29] or rat aorta [30]. Moreover, Kuhlmann et al. [31] showed that quercetin hyperpolarized endothelial cells by activation of BKCa channels. Finally, the flavonoid phloretin activates BKCa channels in myelinated nerve fibres of Xenopus laevis [32].

Therefore, in the present study we investigated whether quercetin activates BKCa currents in rat coronary artery smooth muscle cells (CASM C). We describe that quercetin activates BKCa channels through an increased production of reactive oxygen species (ROS) and this effect plays a role in its coronary vasodilator effect.

2. Materials and methods

The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996).

2.1. Materials

All chemicals and drugs were from Sigma (Alcobendas, Madrid). Quercetin was dissolved in DMSO daily and other drugs in distilled, deionized water.

2.2. Tissue preparation and cell isolation

Coronary arteries (250 – 350 μm internal diameter) were isolated from male Wistar rats (250 to 300 g from our local animal house, Universidad Complutense) in Krebs solution. Unless otherwise stated, arteries were endothelium-denuded. For myocyte isolation, arteries were cut into small segments and placed into a nominally Ca2+-free physiological salt solution (PSS) of the following composition (in mmol/L): NaCl 130, KCl 5, MgCl2 1.2, glucose 10, and HEPES 10 (pH 7.3 with NaOH) containing (in mg/mL) papain 1, dithiothreitol 0.8, and albumin 0.7 for 20 min. Thereafter, artery segments were incubated for additional 15 min in Ca2+-free PSS containing (in mg/mL) collagenase F 1, collagenase H 0.3, and albumin 0.7. Cells were stored in Ca2+-free PSS (4 °C) and used within 8 h of isolation.

2.3. Electrophysiological studies

Membrane currents were measured using the whole-cell configuration of the patch-clamp technique in voltage-clamped CASMC [33]. Currents were normalized for cell capacitance and expressed in pA·pF−1. Membrane potential was measured under current-clamp configuration. K+ currents were recorded using an external solution PSS (see above) containing 1.5 mM Ca2+ and a pipette (internal) solution containing (in mmol/L) KCl 110, MgCl2 1.2, Na2ATP 5, HEPES 10, and EGTA 0.1, pH adjusted to 7.3 with KOH. Current–voltage (I/V) relationships were generated in voltage clamped cells held at a membrane potential of −60 mV and stepped from −60 mV to +60 mV in 10 mV increments. To characterize STOCs the membrane potential of the cells was held at −10 mV.

2.4. Measurement of hydrogen peroxide (H2O2) generation

Endothelium-denuded coronary arteries segments were incubated with the membrane-permeable diacetate form of the dye 2,7-dichlorofluorescein (DCFH-DA, 10 μM for 60–90 min) which is cleaved and trapped intracellularly. ROS in the cells oxidize DCFH, yielding the fluorescent product DCF. Coronary arteries were placed in the stage of a fluorescent inverted microscope (Leica DM IRB, Wetzlar, Germany), superfused with PSS solution (2 ml·min−1) and illuminated through the luminal surface using a 450–490 nm band-pass filter. The emitted fluorescence of the whole artery was filtered using a 515 nm long-pass emission filters.
Images were taken at 2 min intervals with a Leica DC300F color digital camera and saved for off-line analysis. Fluorescence was quantified using ImageJ (ver 1.32j, NIH, http://rsb.info.nih/ij/). Intensity values are reported as a percent of the initial values after subtracting background. Preparations were allowed to equilibrate for 30 min before the application of drugs.

2.5. Contractile tension recording

Rat coronary artery rings (250–350 μm) were fitted with two 40 μm wires and mounted in a small vessel dual chamber myograph for measurement of isometric tension according to Mulvany and Halpern [34]. After a 30 min equilibration period in Krebs Henseleit solution bubbled with a 95% O2–5% CO2 mixture at 37 °C and pH=7.4, segments were stretched to their optimal lumen diameter for active tension development. The absence of a functional endothelium was confirmed by the lack of a relaxant response to acetylcholine.

2.6. Statistical analysis

Data are expressed as means±S.E.M.; n indicates the number of experiments. Statistical analysis was performed using Student’s t test for paired or unpaired observations. Differences were considered statistically significant when P was less than 0.05.

3. Results

3.1. Effects of quercetin on outward currents

Freshly isolated CASMC showed a spindle shape and an average cell capacitance of 22.5±1.1 pF (n=49). Outward currents were recorded including ATP in the pipette solution to minimize the component of ATP-dependent current (IKATP). Under these conditions, when cells were voltage-clamped at −60 mV, the application of 200-ms depolarizing pulses to test potentials from −60 mV to +60 mV in 10-mV increments induced an outward current (Fig. 1A), which activated at potentials positive to −30 mV and was generally reproducible for at least 1 h. Quercetin increased the amplitude of the current in the whole range of test potentials. This effect was significant at concentrations of quercetin N0.1 μM and the maximum increase was observed at 10 μM (Fig. 1C). Moreover, the increase of the current induced by quercetin was more pronounced at −10 mV than at +60 mV.

Fig. 1D shows the time course of the effects of quercetin on the outward current. The response to quercetin reached stable values within 3 to 5 min and was fully reversible after washing in PSS without quercetin. In addition, quercetin (10 μM) caused membrane hyperpolarization of CASMC (Fig. 1E).

The addition of the selective BKCa channel blocker iberiotoxin (100 nM) after quercetin exposure reversed
In another set of experiments, iberiotoxin, added before the exposure to quercetin, significantly reduced the outward current recorded at +60 mV by 42±9% (n=3; P<0.05) and fully prevented the quercetin-induced increase (Fig. 2B and C).

3.2. Effects on STOCs

When CASMC were depolarized from the holding potential of −60 mV to different test potentials, current traces occasionally showed STOCs superimposed on the slowly inactivating outward currents which were more often observed in the presence of quercetin and abolished by 100 nM iberiotoxin (Fig. 3A). In some cells held at −10 mV, STOCs were continuously recorded and addition of quercetin (10 μM) significantly increased their frequency (Fig. 3B and C). Thus, the frequency of STOCs with an amplitude higher than 20 pA increased from 2.2±0.2 Hz (control conditions) to 4.4±0.5 Hz (after quercetin treatment, P<0.01; n=5).

3.3. Role of ROS

In order to analyze the possible role of H₂O₂ in quercetin-induced increase in outward currents, the patch pipette was filled with internal solution containing catalase (100 U ml⁻¹), which catalyzes the conversion of H₂O₂ into H₂O and O₂. The magnitude of the currents in catalase dialyzed CASMC was roughly unchanged as compared to that recorded in the absence of catalase (Fig. 4A vs. Fig. 1B). However, catalase inhibited the increase in outward current induced by quercetin. In fact, in the presence of catalase, the current obtained at potentials positive to +30 mV was significantly reduced by quercetin (Fig. 4A). Furthermore, we also observed that application of t-butyl-hydroperoxide, a membrane permeable hydroperoxide, also produced a marked increase in outward currents in isolated CASMC (Fig. 4B and C). Since modulation of PKC activity has been involved in the responses to both quercetin [35] and hydroperoxide [36], we studied the possible role of this kinase in quercetin-induced increase in outward currents. A

Fig. 2. The selective BKCa channel blocker iberiotoxin prevents and reverts quercetin-induced increase in outward currents in CASMC. (A) Current traces recorded initially under control conditions, then following the addition of quercetin (10 μM), and finally, after the addition of iberiotoxin (IBX) plus quercetin. (B) Current traces recorded initially under control conditions, then following the addition of IBX (100 nM), and finally, after the addition of IBX plus quercetin (10 μM). (C) Current–voltage relationship of the experiments as shown in panel B measured at the end of the 200 ms pulse. Data are means±S.E.M. of 3 cells.

Fig. 3. Quercetin increases the frequency of STOCs in CASMC. (A) Panel shows a STOC during a 200 ms depolarizing step to +60 mV in the presence of quercetin and abolished by the BKCa channel blocker iberiotoxin. (B) Continuous recordings of currents of CASMC held at −10 mV showing STOCs before and during treatment with quercetin. (C) Panel shows an amplification of the recordings shown in panel B.
15 minute perfusion with the PKC inhibitor Gö6976 (0.1 μM) had no effect per se on the currents. Subsequent application of Gö6976 plus quercetin (10 μM) caused an increase in outward currents (230 ±15% at +60 mV, n=4) similar to that observed under control conditions (269 ± 29% at +60 mV, n=8; P<0.05).

We also analyzed whether quercetin produces an increase in cytosolic H₂O₂ using DCF-DA, a cell permeable dye which fluoresces when oxidized by ROS and is a widely used indicator of intracellular H₂O₂. Quercetin, at 1 μM, produced no fluorescence per se in DCF-untreated arteries while concentrations of quercetin ≥ 10 μM produced a clear increase in cell fluorescence (not shown). In DCF-loaded arteries, however, 1 μM quercetin produced a marked increase in fluorescence (Fig. 5). The magnitude and time-course of this increase was similar to that produced by 10 μM t-butyl-hydroperoxide. Quercetin-induced increase in DCF fluorescence was markedly reduced in coronary arteries that were previously incubated for 1 h in the presence of PEG-catalase (300 U ml⁻¹), a membrane permeable analogue of catalase. In another set of experiments, iberiotoxin (100 nM, n=4) did not modify the increase in DCF fluorescence induced by quercetin (87 ±7% of that observed in parallel controls, n=4, measured at 6 min).

3.4. Effects on vascular tone

In order to analyze the role of iberiotoxin-sensitive currents on the coronary vasodilator effects of quercetin, we analyzed the relaxant response to the flavonoid in endothelium-denuded rat coronary arteries. Stimulation with the KV channel blocker 4-aminopyridine (4-AP, 3 mM) resulted in an initial peak followed by a sustained contractile response (76±8% of that produced by 120 mM KCl). Cumulative addition of quercetin in 4-AP-precontracted arteries induced a concentration-dependent relaxant response (Fig. 6A and C). In a parallel set of experiments, rat coronary arteries were treated with iberiotoxin (100 nM) which induced a sustained contractile response and then further stimulated with 3 mM 4-AP which induced a transient contraction (Fig. 6B, final tone of 69±4% of that produced by 120 mM KCl, not different from iberiotoxin-untreated arteries). Under these conditions, the relaxant response of quercetin was significantly inhibited as compared to that obtained in the absence of iberiotoxin (pD₂: control, 5.37±0.09, iberiotoxin, 4.99±0.11, P<0.01, Fig. 6C).

In another set of experiments, the possible role of the endothelium-derived NO in quercetin-induced relaxation was tested in endothelium intact arteries stimulated by 4-AP in the absence or presence of the NOS inhibitor L-NAME (100 μM). In endothelium-intact arteries, the relaxant response of quercetin (pD₂: 5.19±0.08, maximal effect: 99±3%, n=11) was not significantly different to that in denuded arteries. In the presence of L-NAME, the relaxant response (pD₂: 4.91±0.11, maximal effect: 88±2%, n=11) was significantly reduced as compared to that obtained in the absence of L-NAME (pD₂: 5.19±0.08, maximal effect: 99±3%, n=11).
maximal effect 97±1%, n = 10) tended to be reduced but the difference did not reach statistical significance.

4. Discussion

Quercetin and related dietary flavonoids are well known vasodilators in large and small arteries, including coronary arteries [20–24]. However, the mechanisms involved are poorly understood. In the present study, we describe that quercetin increased the amplitude of iberiotoxin-sensitive outward currents in CASMC at concentrations >0.1 μM. Quercetin also increased the frequency of STOCs in depolarized cells. When catalase was present in the internal solution, quercetin failed to increase outward currents. Quercetin also increased the production of H2O2 in coronary arteries as measured by catalase-sensitive DCF fluorescence. The vasodilator effect of quercetin in isolated coronary arteries was inhibited by iberiotoxin.

The relatively uncommon flavonoid phloretin has been reported to activate BKCa channels in myelinated nerve fibres of X. laevis [32]. Herein we show that quercetin, the most abundant flavonoid in our diet, increases outward currents in freshly isolated CASMC. This increase was due to an activation of BKCa channels because it was blocked by the specific BKCa channel blocker iberiotoxin. The effects of quercetin were more pronounced at physiological (negative) potentials. Quercetin also increased the frequency of STOCs which are carried by BKCa and triggered by Ca2+ sparks. Thus, quercetin potentiates a fundamental feedback mechanism in vascular function, limiting membrane depolarization and vasoconstriction. It should be stressed that the effect of quercetin on BKCa currents was already significant at low concentrations, similar to those observed in plasma after the ingestion of quercetin-rich foods (0.1–7 μM, reviewed in Ref. [37]).

Besides the well known oxidative cellular damage, ROS have also been involved in cell signalling in the vasculature [38]. In the coronary arteries, H2O2 has been suggested to play a physiological role as an EDHF [39]. Quercetin is a well known free radical scavenger [40]. However, under certain conditions it may also behave as a pro-oxidant, generating ROS [27,28]. A role for ROS in the Ca2+ signalling in endothelial cells and endothelium-dependent relaxation induced by wine polyphenolics has also been proposed [41,42]. Thus, we tested the hypothesis that quercetin might activate BKCa channels via generating ROS. Specifically, we reasoned that H2O2 might be the likely ROS candidate because it has been reported to activate BKCa currents in coronary artery myocytes [11,43]. Accordingly, we also found that t-butyldihydroperoxide, a membrane permeable hydroperoxide, increased BKCa currents in CASMC. In the presence of catalase, the physiological scavenger of H2O2, quercetin failed to increase BKCa currents suggesting that H2O2 is required for the effects of quercetin.

To confirm that quercetin generated ROS intracellularly in coronary arteries we used DCF, a widely used indicator of ROS production. Quercetin at 1 μM produced no changes in fluorescence in coronary arteries in the absence of DCF which served as a negative control in DCF experiments. This is not a trivial issue because, even when quercetin is weakly fluorescent, this fluorescence is markedly increased when quercetin binds to proteins [44] and precludes the use of high...
concentrations of quercetin in fluorescence studies. Quercetin increased DCF fluorescence and this increase was markedly reduced by PEG-catalase, indicating that it was mainly due to increased cytosolic concentrations of H$_2$O$_2$. Because iberiotoxin has been reported to block NADPH oxidase-induced ROS generation in endothelial cells [45], we also tested whether iberiotoxin could inhibit quercetin-induced ROS generation. The lack of effect of iberiotoxin on quercetin-induced increase in DCF fluorescence further confirms that activation of BKCa is the consequence rather than the cause of ROS generation. The lack of effect of the PKC inhibitor Gö6976 suggests that PKC is not involved in the activation of BKCa currents induced by quercetin.

Moreover, in the presence of catalase, quercetin inhibited the outward current. This reduction was statistically significant at positive potentials (≥+30 mV), far from physiological values, and was not further analyzed in the present study. One possibility is that quercetin might be blocking K$_V$ channels, since we have observed that it reduced the outward currents (carried mostly by K$_V$ channels in Ca$^{2+}$-free solution) in rat pulmonary artery myocytes (Cogolludo, unpublished observations). In addition, quercetin has been reported to inhibit other K$^+$ currents such as HERG currents [46].

In order to test if the activation of BKCa channels had a functional role we analyzed the vasodilator effects of quercetin in coronary arteries. Arterial rings were stimulated by the K$_V$ channel blocker 4-AP to induce a contractile response. This vasoconstrictor was chosen because other typical coronary vasoconstrictors such as KCl, U46619 or 5-HT may block BKCa activity [47,48]. Both 4-AP and iberiotoxin induced a strong contractile response indicating that both channel types regulate membrane potential and tone in coronary arteries but they had no additive effect. The relaxant response induced by quercetin occurred within the same range of concentrations than the activation of BKCa. Furthermore, in the presence of iberiotoxin the concentration–response curve of quercetin was shifted rightwards indicating that BKCa activation plays a role in the vasodilator effects of quercetin. However, at higher concentrations other mechanisms must be also involved. For instance, quercetin, at concentrations ≥10 μM, can induce vasodilation in β-escin-permeabilized rat iliac arteries [21] which is independent of membrane potential and Ca$^{2+}$ entry.

We found no differences when comparing the effects of quercetin in endothelium intact and denuded arteries or in the presence of L-NAME which is consistent with most but not all previous studies in different isolated arteries [20–24]. Kuhlmann et al. [31] showed that quercetin activated the NO/cGMP pathway by activation of endothelial BKCa channels. The activation of BKCa in endothelial [31] and smooth muscle cells (present study) occurs in a similar range of concentrations and might operate simultaneously. However, under our experimental conditions, the endothelium-independent component appears to be sufficient for a full relaxant response.

In conclusion, taken together these present results indicate that quercetin increases BKCa currents via augmented cytosolic H$_2$O$_2$ and this effect is involved in the coronary vasodilator effects of low concentrations of quercetin. Activation of BKCa channels by quercetin may be relevant in the regulation of vascular smooth muscle tone after consuming fruit and vegetables. Furthermore, it may play a role in the antihypertensive effects of quercetin in rat models of hypertension and in the protective effects of quercetin in coronary heart disease as observed in epidemiological studies.

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References


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