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Involvement of thromboxane A_2 in the endothelium-dependent contractions induced by myricetin in rat isolated aorta

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1 The present study was undertaken to analyse the mechanism of the contractile response induced by the bioflavonoid myricetin in isolated rat aortic rings.

2 Myricetin induced endothelium-dependent contractile responses (maximal value = 21 ± 2% of the response induced by 80 mM KCl and $pD_2 = 5.12 \pm 0.03$). This effect developed slowly, reached a peak within 6 min and then declined progressively.

3 Myricetin-induced contractions were almost abolished by the phospholipase A_2 (PLA_2) inhibitor, quinacrine (10 μM), the cyclo-oxygenase inhibitor, indomethacin (10 μM), the thromboxane synthase inhibitor, dazoxiben (100 μM), the putative thromboxane A_2 (TXA_2)/prostaglandin endoperoxide receptor antagonist, ifetroban (3 μM). These contractions were abolished in Ca^{2+}-free medium but were not affected by the Ca^{2+} channel blocker verapamil (10 μM).

4 In cultured bovine endothelial cells (BAEC), myricetin (50 μM) produced an increase in cytosolic free calcium ($[Ca^{2+}]_i$) which peaked within 1 min and remained sustained for 6 min, as determined by the fluorescent probe fura 2. This rise in $[Ca^{2+}]_i$ was abolished after removal of extracellular Ca^{2+} in the medium.

5 Myricetin (50 μM) significantly increased TXB_2 production both in aortic rings with and without endothelium and in BAEC. These increases were abolished both by Ca^{2+}-free media and by indomethacin.

6 Taken together, these results suggests that myricetin stimulates Ca^{2+} influx and subsequently triggers the activation of the PLA_2 and cyclo-oxygenase pathways releasing TXA_2 from the endothelium to contract rat aortic rings. The latter response occurs *via* the activation of T_p receptors on vascular smooth muscle cells.

Keywords: Myricetin; thromboxane A_2; endothelium; rat aorta

Abbreviations: BAEC, bovine aortic endothelial cells; DMSO, dimethylsulphoxide; HBSS, HEPES-buffered saline solution; L-NAME, N^G-nitro-L-arginine-methyl-ester; NO, nitric oxide; PGH_2, prostaglandin H_2; PLA_2, phospholipase A_2; TXA_2, thromboxane A_2; TXB_2, thromboxane B_2

Introduction

Flavonoids are a large group of polyphenolic compounds, diverse in structure and characteristics (Cook & Samman, 1996; Rice-Evans & Miller, 1997). They occur naturally in a variety of foods from vegetable origin, mainly apples, onions, tea and red wine and are an integral part of the human diet (Hertog *et al*., 1993). The estimated diet intake of major flavonoids ranges between 23 and 170 mg day^{-1} (expressed as aglycones) (Hertog *et al*., 1993; Cook & Samman, 1996). In the cardiovascular system, flavonoids have been reported to exhibit antiarrhythmic and antiischaemic effects in the heart (Occhiuto *et al*., 1991). They also produce endothelium-dependent (Andriambeloson *et al*., 1997) and -independent vasorelaxant effects in different blood vessels including the rat thoracic aorta (Duarte *et al*., 1993a,b; Herrera *et al*., 1996) and inhibit platelet aggregation and lipid peroxidation (Tzeng *et al*., 1991; Cook & Samman, 1996). These effects may explain why flavonoid intake appears inversely related with mortality from coronary heart disease in epidemiological studies (Hertog *et al*., 1993).

Myricetin (3,5,7,3′,4′,5′-hexahydroxyflavone) is a major antioxidant food flavonoid which has been shown to exhibit a biphasic contractile response in pre-contracted rat thoracic aorta (Berger *et al*., 1992; Herrera *et al*., 1996). At low concentrations (< 50 μM), it potentiates the responses to different contractile agents such as noradrenaline, high KCl and phorbol 12-myristate 13-acetate in rat aortic rings, whereas at higher concentrations, it exerts a vasorelaxant effect on vessels precontracted with these agents (Herrera *et al*., 1996). The potentiating effect of myricetin has been reported in a number of arteries from different species such as the rat tail and femoral arteries (Berger *et al*., 1992) or the rabbit pulmonary artery (Russell & Rohrbach, 1989). However, the mechanisms involved in the vasoconstrictor response remains unclear.

Therefore, the present study was undertaken to better characterize the contractile effect induced by myricetin in rat thoracic rings. The role of endothelium was investigated. As the endothelium can release different factors including those from the cyclo-oxygenase products, the involvement of this pathway and the underlying mechanism(s) were also studied.

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Methods

Tissue preparation

Wistar rats (either sex, 250–300 g) were killed by a blow on the head. The descending thoracic aorta was quickly dissected and placed in a Krebs' solution of the following composition (mM): NaCl 118, KCl 4.75, $NaHCO_3$ 25, $MgSO_4$ 1.2, $CaCl_2$ 2.0, KH_2PO_4 1.2, and glucose 11. After excess fat and connective tissue were removed, the aortae were cut into rings (2 mm length) and mounted under a basal tension of 2 g in 20 ml organ baths containing Krebs' solution and attached to a force-displacement transducer (Letigraph 2000, Letica) to measure isometric contraction as previously described (Herrera *et al*., 1996). The tissue bath was maintained at 37°C and bubbled with 95% O_2-5% CO_2. For experiments in which Ca^{2+} free Krebs' solution was used, Ca^{2+} was omitted and 0.5 mM EGTA was added. Each preparation was allowed to equilibrate for at least 90 min prior to initiation of experimental procedures and during this period the incubation medium was changed every 20 min. In some experiments, the endothelium was mechanically removed by gently rubbing the ring intimal surface. The presence of functional endothelium was assessed in all preparations by determining the ability of acetylcholine (1 μM) to induce more than 50% relaxation of rings precontracted with phenylephrine (1 μM). Vessels were considered to be denuded of functional endothelium when there was no relaxant response to acetylcholine.

Characterization of the contractile effect of myricetin

After equilibration, aortic rings were challenged with 80 mM KCl Krebs' solution (in which the concentration of NaCl was replaced with an equimolar amount of KCl) in order to test the maximal contractile response of the vessels. Then, rings were washed several times until the contraction had returned to its original baseline. After a 30 min washout period, myricetin was added to the bath in different concentrations (1–100 μM). Preliminary experiments showed that the contractile response to myricetin was not reproducible when it was added in either a cumulative or a non cumulative manner to the bath. Therefore, each vessel was challenged with a single concentration of myricetin.

In another series of experiments, the possible signalling pathways involved in the contractile responses induced by myricetin were analysed in intact aorta. In order to characterize the involvement of the nitric oxide (NO) pathway, some arteries were exposed to the NO synthase inhibitor, N^G - nitro - L - arginine - methyl-ester (L-NAME, 100 μM), added to the bath 30 min prior to myricetin. The sources of Ca^{2+} involved in the contraction induced by 50 μM myricetin, were analysed by testing its effect in Ca^{2+}-free Krebs' solution or in the presence of the calcium channel blocker verapamil (10 μM) added 30 min prior to myricetin. The involvement of arachidonic acid metabolism in the contractile response to myricetin was also analysed. The role of phospholipase A_2 (PLA_2) pathway was investigated using its inhibitor quinacrine (10 μM). The involvement of cyclo-oxygenase pathway was studied using the cyclo-oxygenase inhibitor, indomethacin (10 μM), the thromboxane synthase inhibitor, dazoxiben (100 μM) or the thromboxane A_2 (TXA_2)/prostaglandin endoperoxide receptor blocker, ifetroban (0.3 or 3 μM). All the inhibitors were added to the bath 20 min prior to myricetin (50 μM).

Cell cultures

Bovine aortic endothelial cells (BAEC) were isolated from bovine aortae as described previously by Kessler & Lugnier (1995). Cells were cultured in plastic flasks using as culture medium a mixture of DMEM and HAM F12 mediums (50/50) supplemented with 10% foetal calf serum, 2 mM glutamine, 100 mg ml^{-1} heparin, 10,000 U ml^{-1} penicillin, 10,000 U ml^{-1} streptomycin and 10 μM vitamin C. The cultures were maintained at 37°C in a humidified atmosphere with 5% CO_2. Cells were used for measurements of the cytosolic free Ca^{2+} concentration $[Ca^{2+}]_i$ and the release of thromboxane B_2 (TXB_2) after the first or second passages when confluent.

$[Ca^{2+}]_i$ measurements

$[Ca^{2+}]_i$ measurements were performed with the fluorescent Ca^{2+}-sensitive probe fura-2. Cells were washed and incubated with 5 μM fura-2/AM (the membrane permeant acetoxymethyl-lester derivative) for 1 h at room temperature in HEPES-buffered saline solution (HBSS) of the following composition in mM: NaCl, 119; KCl, 4.75; $CaCl_2$, 1.25; $MgSO_4$, 1.2; KH_2PO_4, 1.2; $NaHCO_3$, 25; glucose, 5 and HEPES 20; pH 7.4. BAEC were then washed twice with phosphate-buffered saline Ca^{2+}/Mg^{2+}-free solution and dispersed using 1% trypsin. After 5 min centrifugation at $60 \times g$, cells were again washed and suspended at a density of 2×10^6 cells ml^{-1} in HBSS. Cells were then transferred to a quartz cuvette in final volume of 2.5 ml, which was constantly stirred and maintained at 37°C. Fluorimetric readings were performed with a F-2000 Hitachi spectrofluorimeter system, with excitation alternating between 340 and 380 nm (10 Hz) and emission at 510 nm. In each preparation, the maximum and minimum fluorescences were sequentially determined by the addition of 10 μM ionomycin in the presence of 2 mM Ca^{2+}, followed by the addition of 10 mM EGTA at pH 8. The $[Ca^{2+}]_i$ was calculated according to the equation described by Grynkiewitz *et al*. (1985).

Thromboxane B_2 production in aortic rings and endothelial cells

To determine the production of TXB_2, rat aortic rings (5 mm length) with and without endothelium were placed in 1 ml of Krebs' solution plus vehicle (DMSO with a final concentration of 0.05% v v^{-1}) or myricetin (50 μM) for 15 min. The tissues were bubbled with a 95% O_2-5% CO_2 gas mixture and kept at 37°C. At the end of this period, 500 μl of the medium was collected and TXB_2 was measured by an enzymeimmunoassay (EIA) (Amersham Life Science, Buckinghamshire, U.K.).

TXB_2 production was assessed in BAEC (10^6 cells ml^{-1}) resuspended in HBSS with or without $CaCl_2$ plus 0.5 mM EGTA. After 15 min incubation with myricetin (50 μM) or myricetin plus indomethacin (10 μM), cells were centrifuged (10 min at 900 r.p.m.) and the medium was collected for TXB_2 measurement by EIA.

Drugs

The following drugs were used: myricetin (Extrasynthese, Genay, France), indomethacin, L-NAME, prazosin, quinacrine and verapamil (Sigma, Madrid, Spain), dazoxiben and ifetroban (Pfizer, New York, U.S.A.). Myricetin was initially dissolved in dimethylsulphoxide (DMSO) to prepare a 10 mM stock solution. Indomethacin was prepared in 2 mM Na_2CO_3 immediately before use and ifetroban was dissolved in absolute

ethanol to prepare a 1 mM stock solution. Other drugs were dissolved in distilled water such that volumes of <0.2 ml were added to the organ chambers.

Statistical analysis

For the contractile experiments, the results were expressed as a percentage of the contractile response induced by 80 mM KCl. Results are expressed as means $\pm$ s.e.mean, n representing the number of aortic rings from different animals. Statistical analysis was performed by means of a two way analysis of variance (ANOVA) followed by a Newman Keuls' test. The differences between control and experimental values were considered significant when $P<0.05$.

Results

Contractile responses

KCl (80 mM) induced a sustained contraction in aortic rings which averaged 831.1 ± 25.9 mg ($n=29$) and 833.3 ± 68.6 mg ($n=9$) in arteries with and without functional endothelium, respectively. In intact rings, myricetin (1–100 μM) induced a concentration-dependent contractile response (pD_2 value $= 5.12 \pm 0.03$), the maximum response was reached at 50 μM ($21.0 \pm 2.3\%$ of the response induced by 80 mM KCl, Figure 1A). As shown in Figure 1B, the increase in tension produced by 50 μM myricetin developed slowly, reached a peak within 6 min and then declined progressively within 20 min. In aortic rings without functional endothelium, myricetin (50 or 100 μM) failed to produce any contraction suggesting that its effect depends exclusively on the presence of functional endothelium (Figure 2). Inhibition of NO-synthase with L-NAME (100 μM) increased by approximately 50% the contractile response to myricetin (Figure 2).

Figure 3 shows that the PLA_2 inhibitor, quinacrine (10 μM), the cyclo-oxygenase inhibitor, indomethacin (10 μM), the thromboxane synthase inhibitor, dazoxiben (100 μM) and the putative TXA_2/prostaglandin endoperoxide receptor antagonist, ifetroban (0.3 and 3 μM) significantly reduced the contractile response induced by 50 μM myricetin.

To study the source of Ca^{2+} involved in the endothelium-dependent contractile response to myricetin, experiments were performed in Ca^{2+}-free medium or in normal medium in the presence of the L-type Ca^{2+} channel blocker verapamil (10 μM). After incubation of aortic rings in Ca^{2+}-free medium the contractile response induced by 50 μM myricetin was abolished. In contrast, verapamil did not significantly affect the contraction induced by myricetin ($21.8 \pm 3.7\%$, $n=10$; $P>0.05$). Moreover, it should be noted that the response to myricetin was not altered in the presence of alpha$_1$ antagonist, prazosin (1 μM) ($18.0 \pm 4.2\%$, $n=6$, $P>0.05$).

$[Ca^{2+}]_i$ measurements in cultured endothelial cells

In fura2-loaded BAEC resuspended in HBSS the basal $[Ca^{2+}]_i$ was 226 ± 37 nM ($n=3$). Myricetin, at concentration at which it produced maximal endothelium-dependent contractile response (50 μM), produced a transient increase in $[Ca^{2+}]_i$ which peaked within 40 s (Figure 4). In Ca^{2+}-free HBSS, the basal $[Ca^{2+}]_i$ was significantly reduced (101 ± 2 nM, $n=4$, $P<0.01$) as compared to the basal level obtained in normal HBSS. Under these conditions, myricetin failed to induce an increase in $[Ca^{2+}]_i$ (Figure 4).

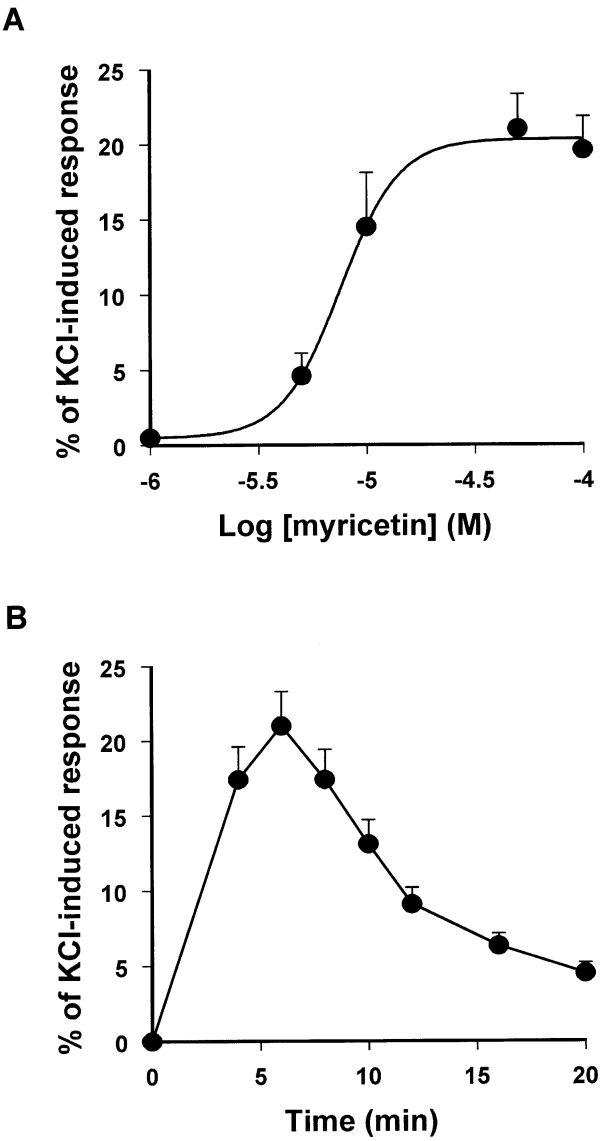

Figure 1 Contractile responses induced by myricetin in endothelium-intact rat aortic rings. (A) The concentration-response curve was constructed in a non cumulative manner by incubation of each ring with a single concentration of myricetin. Each symbol represents the mean of 6–29 arteries; vertical lines show s.e.mean. (B) Time-course of the contractile response induced by 50 μM myricetin. The results in both pannels are expressed as a percentage of the 80 mM KCl-induced contractile response. Each symbol represents the mean of 29 arteries; vertical lines show s.e.mean.

Production of TXB_2

The effects of myricetin on the production of TXB_2 was studied in aortic rings and in BAEC (Figure 5). The basal TXB_2 production was significantly higher ($P<0.05$) in aortic rings with endothelium as compared to that in arteries without endothelium. Myricetin (50 μM) significantly increased the TXB_2 production both in vessels with and without endothelium. However, TXB_2 production was significantly higher ($P<0.01$) in intact vessels treated with myricetin as compared to denuded vessels.

The TXB_2 production in non stimulated BAEC resuspended in HBSS was 4239 ± 453 pg ml^{-1} ($n=5$). Myricetin produced a 5 fold increase in TXB_2 production and this effect was abolished in the presence of indomethacin

(10 μM). In Ca^{2+}-free HBSS, the basal TXB_2 level did not significantly differ to that obtained in normal HBSS. However, under these conditions myricetin did not increase significantly TXB_2 production (Figure 5B).

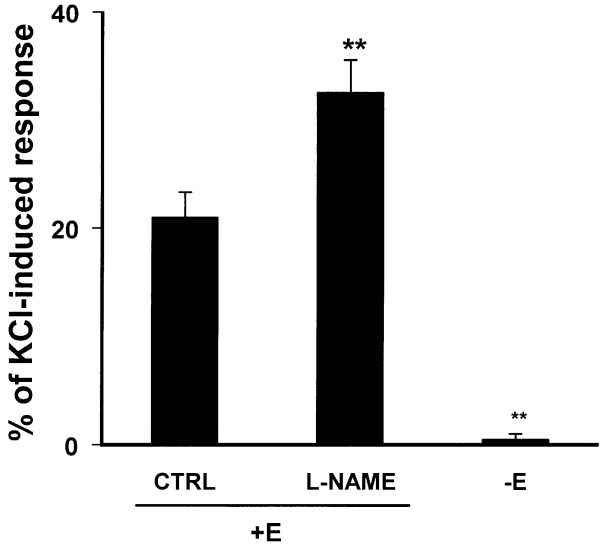

Figure 2 Role of endothelium and nitric oxide in the contractile response induced by 50 μM myricetin. The experiments were performed in endothelium-intact (+E) or endothelium denuded (−E) aortic rings incubated in the absence (CTRL) or in the presence of L-NAME (100 μM) for 20 min before the addition of 50 μM myricetin. The results are expressed as a percentage of the 80 mM KCl-induced contractile response. Each symbol represents the mean of 6–29 arteries; vertical lines show s.e.mean. $^{**}P<0.01$ vs CTRL.

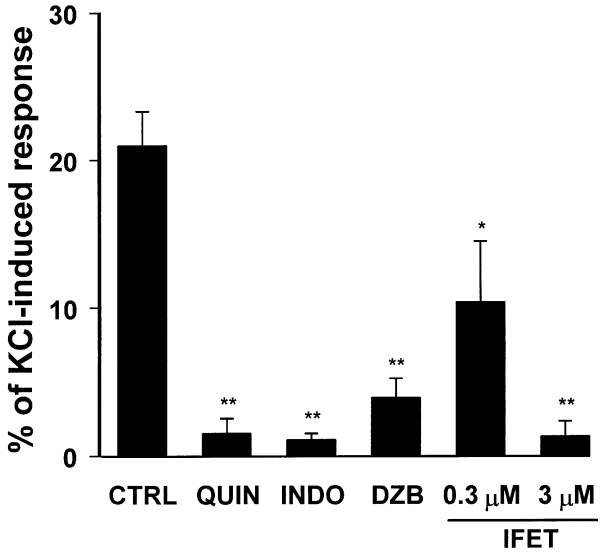

Figure 3 Effects of arachidonic acid pathway inhibitors on the contractile response induced by 50 μM myricetin. Aortic rings with functional endothelium were incubated in the absence (CTRL) or in the presence of quinacrine (QUIN, 10 μM), indomethacin (INDO, 10 μM), dazoxiben (DZB, 100 μM) or ifetroban (IFET, 0.3 or 3 μM) for 20 min before the addition of myricetin. The results are expressed as a percentage of the 80 mM KCl-induced contractile response. Each symbol represents the mean of 6–29 arteries; vertical lines show s.e.mean. $^{*}P<0.05$, $^{**}P<0.01$ vs CTRL.

Discussion

The present study indicates that the contraction of isolated rat aorta induced by myricetin requires the presence of endothelium. The results provide evidence that myricetin, through the activation of phospholipase A_2 pathway, induces the release of TXA_2 following the activity of cyclo-oxygenase eliciting vascular smooth muscle contraction subsequent to T_p receptor stimulation. Likewise, myricetin is able to release TXA_2 through a cyclo-oxygenase inhibitor sensitive pathway in cultured endothelial cells. All of the above mechanisms implicate an increase of $[Ca^{2+}]_i$ in the endothelial cells through an extracellular Ca^{2+}-dependent pathway.

The endothelium contributes to the local regulation of vascular smooth muscle tone by releasing endothelium-derived relaxing factors (NO, prostacyclin and endothelium-derived hyperpolarizing factor) and endothelium-derived contracting factors such as endothelins, vasoconstrictor prostanoids and superoxide anions (Moncada *et al*., 1991; Luscher & Barton, 1997). The contractions induced by myricetin were abolished in endothelium denuded rings indicating that myricetin either removed an endothelium derived vasodilator or released an endothelium-dependent vasoconstrictor. The augmented contractile responses to myricetin in the presence of the NO synthase inhibitor L-NAME indicates that these responses are not mediated by inhibition of NO synthesis and supports the idea that basal or myricetin-induced NO release partly inhibits the increase of vascular tone induced by myricetin. Cleavage of membrane lipids by PLA_2 causes the release of arachidonic acid that can be metabolized *via* the cyclo-oxygenase and TXA_2 synthase enzymes into TXA_2 (Mentha & Roberts, 1983). TXA_2 is produced mainly by the media layer of intact blood vessels (Brunkwall *et al*., 1987), although some is synthesized by vascular endothelial cells (Ingermam-Wojenski *et al*., 1981). The contractions induced by myricetin were inhibited either in the presence of the phospholipase A_2 inhibitor, quinacrine, or the cyclo-oxygenase inhibitor, indomethacin. These findings suggest that the metabolism of arachidonic acid through the cyclo-

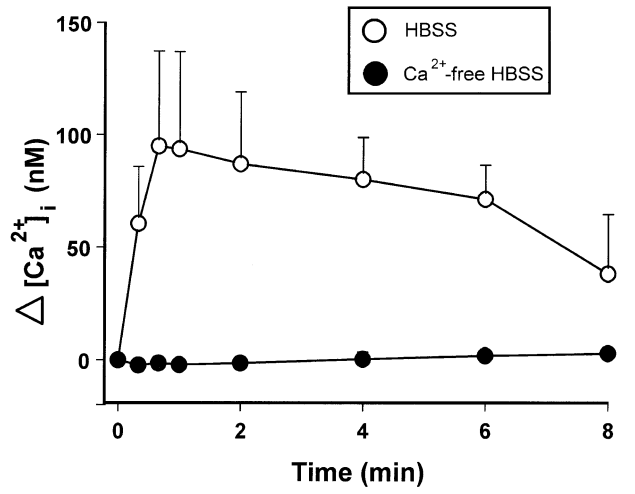

Figure 4 Time course of the effects of 50 μM myricetin on intracellular calcium measured in fura2-loaded BAEC resuspended in HBSS or in Ca^{2+}-free HBSS. Results are expressed as increases in $[Ca^{2+}]_i$ above baseline. Each symbol represents the mean of 3–4 experiments; vertical lines show s.e.mean. Myricetin was added at time 0.

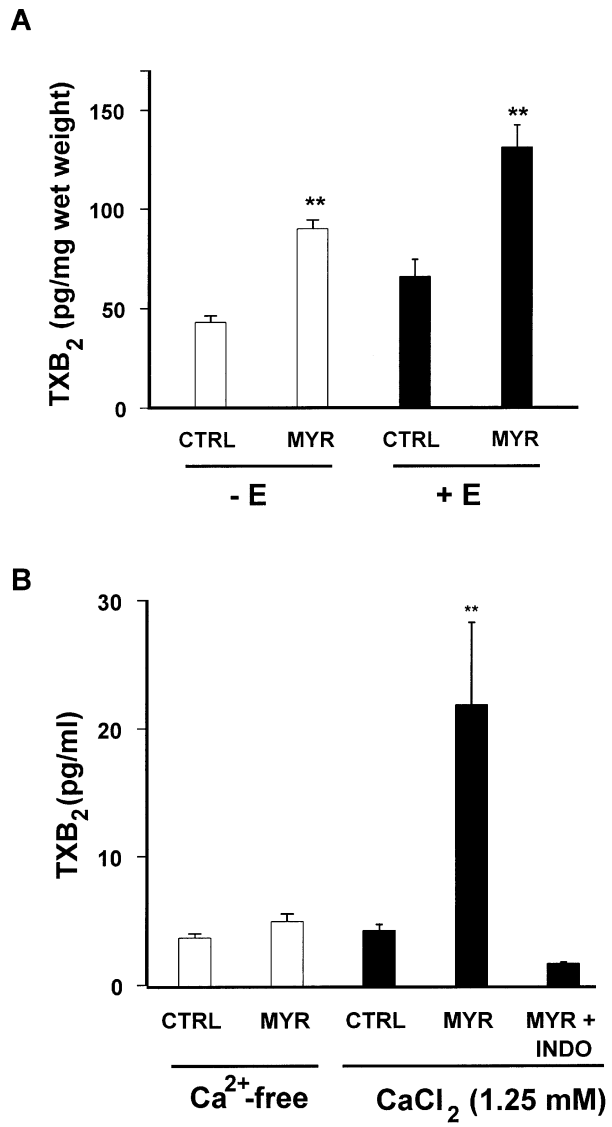

Figure 5 TXB_2 production stimulated by myricetin in (A) aortic rings and (B) BAEC. (A) shows the TXB_2 production in resting (CTRL) or myricetin-stimulated (50 μM for 15 min, MYR) aortic rings with (+E) or without (−E) endothelium. Each column represents the mean of six arteries; vertical lines show s.e.mean. $^{**}P<0.01$ vs CTRL. (B) shows the TXB_2 production in BAEC resuspended in HBSS or a Ca^{2+}-free HBSS. BAEC were incubated for 15 min in the absence (CTRL) or in the presence of myricetin (50 μM, MYR) or myricetin plus indomethacin (10 μM, INDO). Each column represents the mean of five experiments; vertical lines show s.e.mean. $^{**}P<0.01$ vs CTRL.

oxygenase pathway plays a key role in the endothelium-dependent responses to myricetin. Prostaglandin H_2 (PGH_2), the unstable precursor of prostaglandin $F_{2\alpha}$, prostaglandin E_2 and TXA_2 induces a contractile response in rat aorta (Förstermann *et al*., 1984). In the present study, we found that the activation of TXA_2-PGH_2 receptors on vascular smooth muscle is implicated in the endothelium-dependent contractions induced by myricetin. This conclusion is based on the observation that the TXA_2-PGH_2 receptor antagonist ifetroban (Ogletree *et al*., 1992) abolished this contractile response. Moreover, we found that myricetin, at a concentration at which it induces its maximal contractile response, stimulated TXB_2 production in intact aortic rings and cultured BAEC. However, we found that TXB_2 accumulation was only decreased by 50% by endothelial denudation while the contractile responses were abolished. The thromboxane synthase inhibitor dazoxiben partly inhibited myricetin-induced contractions suggesting that TXA_2 is involved in these effects. However, PG endoperoxides (PGG_2 and PGH_2) released from the endothelium may also participate, accounting for the endothelial dependence of the contractile response. A number of reports have shown that flavonoids can modulate arachidonic acid metabolism (for a review see Alcaraz & Ferrándiz, 1987). While most flavonoids were able to inhibit both the platelet cyclo-oxygenase and lipoxygenase pathways at relatively high concentrations (50 μM), only myricetin (10 μM) increased the conversion of arachidonic acid into TXB_2 (Landolfi *et al*., 1984). Furthermore, we cannot exclude that an additional source of TXA_2 in our study is from platelets adhered to the intact aorta and that physical endothelium-denudation would also remove the platelets.

The contractile response induced by myricetin was abolished in aortic rings incubated in a Ca^{2+}-free medium. Extracellular Ca^{2+} may be required either for smooth muscle contraction or for endothelial production of TXA_2. Activation of TXA_2-PGH_2 receptors by TXA_2 mimetics, such as U46619, produces vascular smooth muscle contractions by increasing the calcium sensitivity of the contractile apparatus through an activation of protein kinase C with no significant change or relatively small increases in $[Ca^{2+}]_i$ (Himpens *et al*. 1990; Jiang *et al*., 1994). Thus, an extracellular source of Ca^{2+} does not appear to be a requisite for TXA_2-induced contraction. Moreover, PLA_2 isoforms involved in signal transduction are regulated by $[Ca^{2+}]_i$ (Kramer & Sharp, 1997) and, therefore, an increase in $[Ca^{2+}]_i$ in the endothelial cells might be the triggering signal for myricetin-induced TXA_2 production. In fact, myricetin increased $[Ca^{2+}]_i$ in BAEC. Furthermore, in Ca^{2+}-free solution myricetin failed to increase both $[Ca^{2+}]_i$ and TXB_2 levels, indicating that myricetin stimulated extracellular Ca^{2+} influx into the endothelial cells rather than its release from intracellular stores. The inability of verapamil to inhibit myricetin evoqued contractions suggests that myricetin stimulates Ca^{2+} entry to endothelial cells through a pathway insentitive to this L-type Ca^{2+} channel blocker.

To the best of our knowledge, there is no information concerning the bioavalability and plasma levels of myricetin in humans. However, myricetin is present in the diet in smaller amounts than the more common flavonoid quercetin which is detected as such or as conjugated active metabolites in plasma from non-supplemented humans at concentrations up to 1.6 μM (Paganga & Rice-Evans, 1997). Thus, it is unlikely that subjects on a normal diet reach plasma concentrations of myricetin as high as those used in the present study. However, it cannot be excluded that these levels might be reached after selected meals (e.g. broad beans and red wine) with a high content of myricetin.

In conclusion, our findings suggest that an activation of PGH_2-TXA_2 receptors on vascular smooth muscle by the TXA_2 released from endothelium by a Ca^{2+}-sensitive activation of the arachidonic acid metabolism is the main mechanism involved on the contractile response induced by myricetin in rat aortic rings.

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