Chronic administration of quercetin induces biomechanical and pharmacological remodeling in the rat coronary arteries

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Short title: Quercetin-induced remodeling of coronary arterioles

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Summary

Acute dilation brought about by the dietary flavonoid quercetin in coronary arterioles has been described earlier, but no information is available on its chronic effects. Male Wistar rats (body weight about 190 g) were divided to two groups: the quercetin-treated group (n=22) had quercetin supplementation of approximately 30 mg/kg/day, whereas the control group (n=20) had none. After eight weeks of treatment, intramural coronary arterioles with identical passive diameters (178±14 and 171±9 μm) were prepared and their biomechanics and pharmacological reactivities were tested using pressure arteriography *ex vivo*. The spontaneous tone of quercetin-treated arteries was higher (16.5±1.9% vs. 12.9±0.9%), which resulted in a reduced lumen size (144±9 μm vs. 167±12 μm), thicker vascular wall (22.6±1.8 μm vs. 17.4±1.6 μm) and decreased tangential wall stress (16.8±1.1 kPa vs. 20.5±1.6 kPa) in supplemented animals (in spontaneous tone at 50 mmHg, $p<0.01$ in all these comparisons). Elevated basal NO release resulted in increased endothelial dilation in quercetin-treated animals, especially at higher intraluminal pressures (10.8±2.5% vs. 5.7±1.3% at 70 mmHg, $p<0.01$). We found remodeling of the geometry of coronary arterioles to ensure higher dilatory reserve and nitrogen monoxide production, as well as lowered elastic stress of the vessel wall.

Keywords: quercetin, vascular remodeling, coronary circulation, arterioles, endothelial nitric oxide synthase

Abbreviations. nKR: normal Krebs-Ringer solution, Ca^{2+}free: Ca^{2+}-free Krebs-Ringer solution, Ach: acetylcholine, BK: bradykinin, L-NAME: nitro-L-arginine methyl ester, NO: nitric oxide, eNOS: endothelial nitric oxide synthase, ROS: reactive oxygen species

Introduction

Dietary polyphenols are present in a mixed human diet in remarkable amounts, around 1 g/day. They are represented by diverse molecules, quercetin being one of the most frequent components among them (28-42 mg/day) (Edwards *et al.* 2007, Scalbert and Williamson 2000). This amount has cardioprotective, antihypertensive (Larson *et al.* 2012), antioxidant (Galisteo *et al.* 2004) and antilipemic (Lee *et al.* 2011) effects. Proper functioning of coronary resistance arteries is a key condition to supply the myocardium with oxygen and nutrients. Although the acute dilating effect of quercetin on major and resistance-sized vessels including coronary resistance arteries (Ibarra *et al.* 2003, Monori-Kiss *et al.* 2014) has been proven earlier, much less is known about the chronic effects of quercetin on resistance arteries. No publication deals with coronary resistance arteries in this respect, despite the accepted view that quercetin is a potent preventive and therapeutic substance for several forms of cardiovascular disease, including cardiac hypertrophy (Yan *et al.* 2013, Han *et al.* 2009). Targeted chronic remodeling studies on large arteries have shown decreased neointima formation and decreased collagen deposition in the abdominal aorta (Huang *et al.* 2009) as well as decreased collagen I and III expression in myocardial tissue (Yan *et al.* 2013). These results raise the possibility but do not prove that chronic quercetin supplementation might also favorably affect the biomechanical properties of resistance-sized arteries. Whereas there are several differences between human and rodent metabolism, oral administration of quercetin to rodents seems to be a good model for polyphenol-rich food in humans (Kawai *et al.* 2009).

The aim of this study was to examine the long-term effects of a realistic dose of the flavonoid quercetin on the segmental remodeling of biomechanical and pharmacological properties of coronary arterioles compared to those from untreated normal control rats kept in parallel. It is hypothesized that long-term quercetin-treatment enhances basal NO-mediated dilation, limits dilation to norepinephrine and improves adaptive function of smooth muscle.

Materials and Methods

1. Animal treatment and preparation of the segments

All procedures conformed to the Guide for the Care and Use of Laboratory Animals (Guide for the care and use of the laboratory animals, 8th edition, ELAR/NRC 2011), the legal and institutional guidelines for animal care and were approved by the Animal Care Committee of the Semmelweis University and Hungarian authorities (22.1/2960/003/2009). Male Wistar rats at the age of approximately 2 months (180-200 g body weight) were randomly distributed to two groups. All animals had the same rat chow ad libitum (S8106-S011 SM, Ssniff Spezialdiaten). The quercetin-treated group (n=22) had this standard rat chow and a suspension of quercetin, 0.3 g/liter suspended in tap water, to drink ad libitum. After sterilization in autoclave the chow is almost quercetin-free (Yoo *et al.* 2012). Based on an average of 100 ml pro kg body weight water consumption (Wade *et al.* 2002), this treatment means approximately 30 mg/ kg body weight of quercetin supplementation pro day. Considering the higher metabolic rate per kg body weight of rats, this dose is comparable with 5 mg/kg/day human dose (Reagan-Shaw *et al.* 2008). A suspension of this concentration was prepared of quercetin hydrate (IUPAC name of quercetin: 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4*H*-chromen-4-one, purity ≥95% measured by HPLC analysis performed by manufacturer) without any excipient. Suspensions proved to be stable. To prevent oxidation, the suspension was freshly prepared on every second day. The control group (n=20) was kept in parallel (at the same temperature and room) on the same common chow but provided with tap water (vehicle of the suspension) without any supplementation. The animals' weight, turgor of the skin and behavior were checked twice a week.

After 8 weeks of treatment, rats were anesthetized with pentobarbital (Nembutal, Ceva, 45 mg/kg body weight i.p.). The heart was removed, its weight was measured, and was

next put in cold oxygenized Krebs-Ringer solution (composition in mmol/liter: NaCl 119, KCl 4.7, NaH_2PO_4 1.2, $MgSO_4$ 1.17, $NaHCO_3$ 24, $CaCl_2$ 2.5, glucose 5.5, and EDTA 0.034). A small intramural coronary arteriole with an outer diameter between 150-200 μm was prepared *in situ* from a terminal branch of the left anterior descendent coronary artery, as described earlier (Nadasy *et al.* 2001). The excised vessel segments had more than 2.0 mm length to maintain the physiological cylindrical shape. They were cannulated at both ends using microcannulas with outer diameters around 130 μm, and mounted in a glass-bottomed organ bath (Experimetria LTD), then axially extended by 10 %, to simulate the *in vivo* axial extension ratio. Artery segments were pressurized intraluminally by servo-controlled pumps (Living Systems). The bath was thermostated at 37 ^{o}C, and bubbled with a gas mixture of 5% CO_2, 20% O_2 and 75% of N_2, keeping the pH at 7.4. During incubation, continuous superfusion was ensured at a rate of 2.8 ml/min, whereas the bath volume was 12.0 ml. The organ bath was positioned on the stage of an inverted microscope (Leica), where pictures of the arteries were taken by a digital camera (Leica DFC 320). Pictures were analyzed offline (Leica Qwin), where inner and outer diameters were measured. Calibration was made using a micrometer etalon (Wild). All chemicals (acetylcholine chloride, L-norepinephrine hydrochloride, nitro-L-arginine methyl ester hydrochloride (L-NAME), bradykinin acetate (purity of all chemicals over 98%) and quercetin hydrate (purity over 95%)) were purchased from Sigma-Aldrich.

2. *Ex vivo* protocols

To study the biomechanical properties of coronary resistance artery segments, two protocols were used. In the first series of experiments, arteries from 12 quercetin-treated animals and 10 control rats were taken and incubated in nKR solution at 50 mmHg intraluminal pressure for 30 minutes. Arteries develop spontaneous tone under these conditions. A pressure diameter curve was next determined by raising the pressure from 10 to

100 mmHg in 10 mmHg steps with a 3-minute incubation at each pressure. After a 10-minute rest the original diameter was restored, and we added norepinephrine to the bath (final concentration 10 µmol/liter), incubated the vessel for 10 minutes and pressure diameter curves were repeatedly recorded. Without washout, we added 10 µmol/liter acetylcholine, incubated for 10 minutes, and repeated the pressure diameter curve. 100 µmol/liter L-NAME was next added to block NO synthesis. After 20 minutes of incubation, a pressure diameter curve was taken again. To test reproducibility, a washout with nKR solution was made, followed by incubation for 20 minutes. Vessels with spontaneous tone differing from the original by more than 5% at this point were rejected. Finally, the superfusion was changed to Ca^{2+}-free Krebs-Ringer solution (composition in mmol/liter: NaCl 92, KCl 4.7, NaH_2PO_4 1.18, $MgCl_2$ 20, $MgSO_4$ 1.17, $NaHCO_3$ 24, glucose 5.5, EGTA 2, and EDTA 0.025), and after 20 minutes of incubation the passive pressure diameter curve was recorded.

In the second series, we investigated the properties of artery segments from 10 animals from both groups. The protocol started with a 30-minute incubation at 50 mmHg in nKR solution, and a pressure diameter curve was next taken as described earlier. After a 10-minute rest, bradykinin was added to the bath in a concentration of 1 µmol/liter, and arterioles were incubated for 10 minutes before the pressure diameter curve was repeated. Bradykinin was then washed out, the original tone was checked, and L-NAME was added in 100 µmol/liter concentration. The incubation time was 20 minutes, similarly to the first protocol, and pressure diameter curves were taken again. Reproducibility was tested by measuring spontaneous tone in nKR solution at 50 mmHg; the superfusion was next changed to Ca^{2+}-free Krebs-Ringer, and the passive curve was recorded.

3. Calculation formulas

$D_{i\ (actual)}$ is the inner diameter in µm at the actual pressure and in solution. $D_{i\ (passive)}$ is the inner diameter (µm) measured in Ca^{2+}-free Krebs-Ringer solution at the given pressure.

Actual diameters were normalized for the passive conditions at each pressure level (% of Ca^{2+}-free= $(D_{i\ actual}*100)/D_{i\ passive}$). Spontaneous tone was calculated as percentage of Ca^{2+}-free diameter (spontaneous tone (%) = 100-inner diameter (% of Ca^{2+}-free)). Calculation of wall stress was based on the Laplace-Frank equation ($\sigma = (P_t*r_i)/h$), where P_t is the transmural pressure (in this case, the intraluminal pressure), r_i is the inner radius in µm, and h is wall thickness in µm. Calculation of incremental elastic modulus was based on Cox's formula (Cox 1979) (E_{inc} (kPa) = $[(2r_i^2*r_o)*\Delta P]/[(r_o^2-r_i^2)*(r_{2o}-r_{1o})]$), where r_i is the inner radius in µm at lower pressure, r_o is the outer radius in µm at lower pressure, ΔP is the increase in pressure (in this case ΔP = 10 mmHg = 1,33 kPa), r_{2o} is the outer radius at higher pressure and r_{1o} is the outer radius at lower pressure.

4. Statistical analysis and data presentation

Data recording and calculations were made with Microsoft Excel. Statistical analysis was performed with GraphPad Prism 6. Values are expressed as mean, with the standard error of mean included. Statistical comparisons were made using one or two-way ANOVA with Bonferrroni post-hoc test, and linear regression. Statistically significant differences were accepted with p values less than 0.05.

Results

Both groups were healthy, the behavior, alertness, movement, hair or body position of the animals did not show any difference. The average body weight at the beginning of the treatment was 194±4 g. After 8 weeks, no difference was found either in body weights (507±9 g, vs. 504±10 g) or heart weights (1.38±0.14 g vs. 1.35±0.18 g) between the control and quercetin-supplemented groups, respectively.

As described in Methods, coronary resistance arteries with close to identical outer diameters during preparation have been selected. Fig.1.A shows that there was no difference in fully relaxed inner diameters measured in Ca^{2+}-free solutions (passive diameter). The passive incremental elastic moduli did not differ either (Fig 1.B). However, inner diameters in oxygenized 37 ^{o}C warm Krebs-Ringer solution were significantly smaller in quercetin-treated animals (Fig 2.A) as a result of elevated spontaneous myogenic tone, characteristic for intramural coronary arterioles (Fig 2.B, $p<0.01$). This also resulted in a thicker vascular wall under active conditions (Fig. 2.C). Under passive conditions, wall thicknesses and relaxed diameters were practically identical (Fig 2.D, $p<0.01$). In turn, this increase in vessel wall thickness resulted in decreased tangential wall stress in quercetin-treated animals under active conditions (Fig 2.E, $p<0.01$). Applying linear regression we found significant difference between slopes (95% confidence intervals: control 0.3896 to 0.4821, quercetin treated 0.3163 to 0.3865, $p=0.004$).

We found a slight but significant reduction in vasodilatory response to 10 μmol/liter norepinephrine in the range of 10 to 80 mmHg ($p=0.01$, Fig 3) in quercetin-treated animals. Dilation rather than constriction is the typical response to norepinephrine in resistance-sized coronary arterioles (Ming *et al.* 1997). At the same time, acetylcholine (10 μmol/liter) and bradykinin (1 μmol/liter) induced dilation have also been somewhat reduced (Fig 4.A and Fig 4.B), However, administration of L-NAME (100 μmol/liter) induced much more forceful contractions in quercetin-supplemented animals, revealing that basal NO-dependent vasodilation was much higher in them (between 60-100 mmHg, $p<0.03$, Fig 4. C). Fig. 4.D. reveals that while there is no difference in maximum endothelial dilation capacity, a higher part of this capacity is used under basal conditions in quercetin-supplemented animals' vessels.

Discussion

In this study we identified the effects of a 30 mg/kg/day supplementary dose of quercetin compared to those of the routine quercetin intake with standard rat chow, on the passive and active biomechanical properties, and on some of the pharmacological responsiveness of intramural coronary arterioles of the rat. Passive properties such as wall thickness and passive elasticity did not change due to quercetin supplementation for eight weeks in comparison with the control. However, characteristic remodeling of the active biomechanical and the pharmacological properties could be observed. Spontaneous tone increased and caused reduced lumen and increased vascular wall thickness in spontaneously contracted arteries, ensuring a higher dilatation reserve for these arteries. In parallel with this, a significantly elevated basal endothelial dilation of the arteries from quercetin-supplemented animals was found.

In an earlier report, quercetin supplementation resulted in improvement of wall elasticity in abdominal aortas denuded by a balloon catheter (Huang *et al.* 2009). No change in passive segmental geometry or passive elastic properties was found in our experiment on coronary arterioles. This can be explained by the use of a lower amount of quercetin in our studies. We applied a 30 mg/kg daily dose in contrast to the evidently pharmacological doses of 100 mg/kg and 200 mg/kg used by the above cited authors. This dose of 30 mg/kg/day in rats is thought to be comparable with 5 mg/kg/day in humans (Reagan-Shaw *et al.* 2008), a dose commonly advised for human nutrition studies (McAnulty *et al.* 2013, Perez *et al.* 2014). Furthermore, we must not forget that our studies were made in otherwise healthy vessels, not on pathologic large artery specimens.

Increased myogenic tone is one of our key observations. It can be the result of altered calcium homeostasis in smooth muscle cells by quercetin acting as an activator of L-type Ca^{2+} channels (Saponara *et al.* 2002), and also having a biphasic effect on Ca^{2+} ATP-ase (McKenna *et al.* 1996). The tone elevation we observed may have two consequences. First, *in*

situ lumen size decreases. That means that despite the identity of passive vessel characteristics, the position in the coronary network of the quercetin-treated artery segments we prepared was different from that of the control artery segments with the same passive diameter. For example, at 70 mmHg intraluminal pressure the inner diameter of treated arteries was 147±10 μm, whereas that of the untreated ones was 172±13 μm ($p<0.05$ with Bonferroni post hoc test). This difference means that upon full relaxation (supposing other parameters are unchanged) there is a 75.2% elevation in flow in the control, and a 101.6% elevation in blood flow in quercetin-treated arteries (computation based on the Poiseuille law). We can declare that the quercetin-treated arteries had a much higher dilatation reserve for coronary vasomotion. The second difference concerns *in situ* wall thickness. At 70 mmHg intraluminal pressure arteries in spontaneous contraction had a wall thickness of 16.4±1.6 μm, whereas in quercetin–treated arteries 22.3±2.0 μm wall thickness was observed. These values correspond to tangential wall stresses of 30.9±2.5 kPa in control vessels, and to 24.5±1.8 kPa in quercetin-treated arteries at 70 mmHg intraluminal pressure under spontaneous myogenic tone ($p<0.05$). There is good reason to assume that quercetin-treated arteries function at much lower wall stresses than control ones *in vivo*.

Type β2 adrenergic receptors prevail on smooth muscle cells of resistance-sized coronary arteries; the direct effect of norepinephrine on these vessels is relaxation (Ming *et al.* 1997). In our experiments, both control and quercetin-treated arteries are relaxed by norepinephrine, the latter group producing less extensive relaxation. This is in good agreement with recent observations, according to which quercetin, its glucosides and its 3-glucuronide metabolite inhibit the activity of the enzyme adenylyl cyclase (Yamazaki *et al.* 2014, Pavan *et al.* 2015). After oral administration quercetin is metabolized to sulphates and glucuronides by the liver both in humans (Ishizawa *et al.* 2011) and in the rat (Omar *et al.* 2014). A β-glucuronidase can cleave quercetin from the metabolite in the vessel wall (Perez-

Vizcaino *et al.* 2012), thus these compounds can affect adenylyl cyclase, causing limited relaxation. Interestingly, recent studies indicate the haemodynamic effect of 3-(3-hydroxyphenyl)propionic acid, produced by the human colon microflora from quercetin (Najmanová *et al.* 2016).

One important observation of our experiments was that chronic supplementation of quercetin enhanced NO-mediated dilation as shown by the L-NAME contractions, especially at higher intraluminal pressures. Because quercetin supplementation does not increase eNOS expression in healthy rodents (Takahashi *et al.* 2015, Wan *et al.* 2009), this could be the consequence of two mechanisms. First, quercetin induces rapid phosphorylation of eNOS at serine 1179, which in turn increases the activity of the enzyme (Li *et al.* 2012). Another mechanism can be an enhanced Ca^{2+}-entry into endothelial cells and consequent elevated NO production involving large conductance Ca^{2+}-activated K^{+} channels (BK(Ca) channels) (Kuhlmann *et al.* 2005). As it is summarized in Fig.4D, the higher basal NO release of supplemented arteries (L-NAME effect) could not be further increased by eNOS activators (Ach, BK), and this explains the reduced effect of these substances shown in Fig.4.A and Fig.4.B. Direct measurements on smooth muscle cell calcium homeostasis and endothelial expression of eNOS were not made. These conclusions are based on literature, because of available data in published studies.

Some limitation of our study seems to be the precise dosage of quercetin. Taking into consideration of the long animal treatment period, we choose the safer dosage by drinking water instead of gavage. In both groups, 3 animals were kept in a cage to minimize non-specific stress. With standardizing the environment, we ensured standard and average water consumption during treatment, which provided a fairly standardized quercetin intake.

In conclusion, chronic administration of quercetin to rats in dietetically real amounts induces a structural and functional remodeling of resistance coronary artery segments, including reduced the elastic stress of the vessel wall, increased dilatory reserve, and augmented NO-mediated endothelial dilation. Quercetin-treatment results in a substantially higher (30%) spontaneous tone. The enhanced basal NO-mediated dilation and higher spontaneous tone together may provide a new balance point of vasodilatory and vasoconstrictor mechanisms. Although polyphenols are not vitamins (Vickery *et al.* 1950), long-term quercetin intake may result in wider adaptation range and lower elastic stress for coronary arteries.

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Conflict of interest

No conflicts of interest, financial or otherwise, are declared by the authors.

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Legends of figures

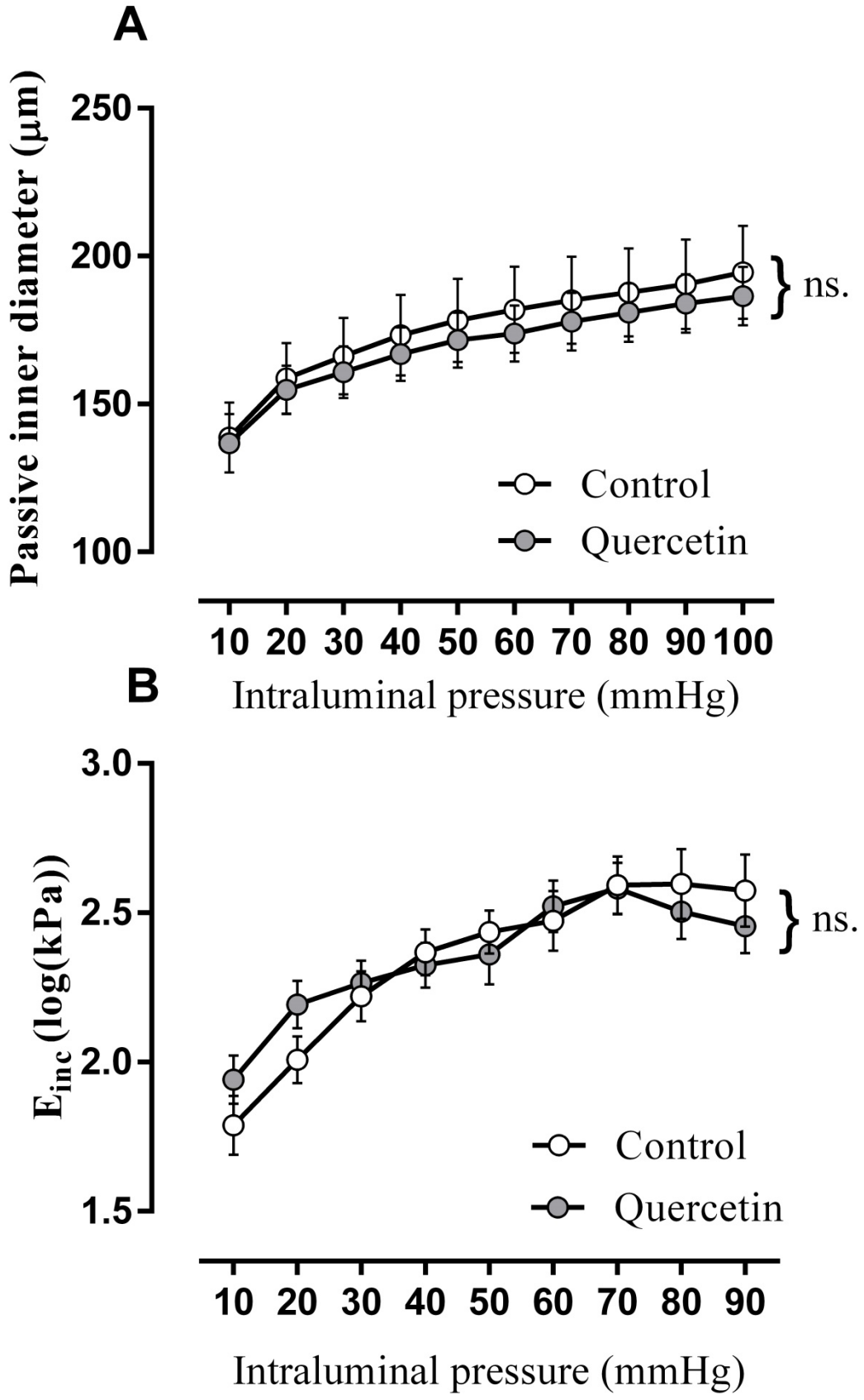
Figure 1. Passive biomechanical properties. Panel A. Passive inner diameter of coronary arterioles at different intraluminal pressures. There is no statistically significant difference between the two groups in passive lumen geometry with two-way ANOVA. Result of both protocols (n=22 quercetin-treated and n=20 control). Panel B. Incremental elastic modulus of passive segments as a function of intraluminal pressure. There is no significant difference in passive elasticity between the two groups (Two-way ANOVA, n=22 quercetin-treated and n=20 control).

Figure 2. Active biomechanical properties. Panel A. Pressure diameter curves of coronary segments from control and quercetin-treated groups in oxygenized nKR solution (segments in spontaneous and myogenic tone). Note that segments from the quercetin-treated group developed higher spontaneous tone, and thus had decreased inner diameter under active conditions. (Two way ANOVA, $p<0.01$; n=22 quercetin-treated and n=20 control) Panel B. Spontaneous and myogenic tone developed in nKR in response to stepwise elevation of intraluminal pressure. Note that the quercetin-treated group had increased spontaneous tone. Data expressed in percent of passive diameter. (Two-way ANOVA, $p<0.01$; n=22 quercetin-treated and n=20 control) Panel C. Wall thickness under active conditions, measured in nKR solution. In parallel with the higher tone, the vessel wall was thickened. (Two-way ANOVA, $p<0.01$; n=22 quercetin-treated and n=20 control) Panel D. Comparison of wall thicknesses at 50 mmHg intraluminal pressure in passive and spontaneously contracted arteries, revealing remodeling of the wall under active conditions. (One-way ANOVA, $p<0.05$; n=22 quercetin-treated and n=20 control) Panel E. Tangential wall stress under active conditions (in nKR solution). Note reduced wall stress in the arteries of quercetin-treated animals. (Two-way ANOVA, $p<0.01$; n=22 quercetin-treated and n=20 control)

Figure 3. Dilation induced by 10 μmol/liter norepinephrine (as compared to segments in myogenic tone in nKR solution). See the reduced beta adrenergic dilation of quercetin-treated segments. Statistically significant between 10-80 mmHg intraluminal pressure. (Two-way ANOVA $p<0.01$)

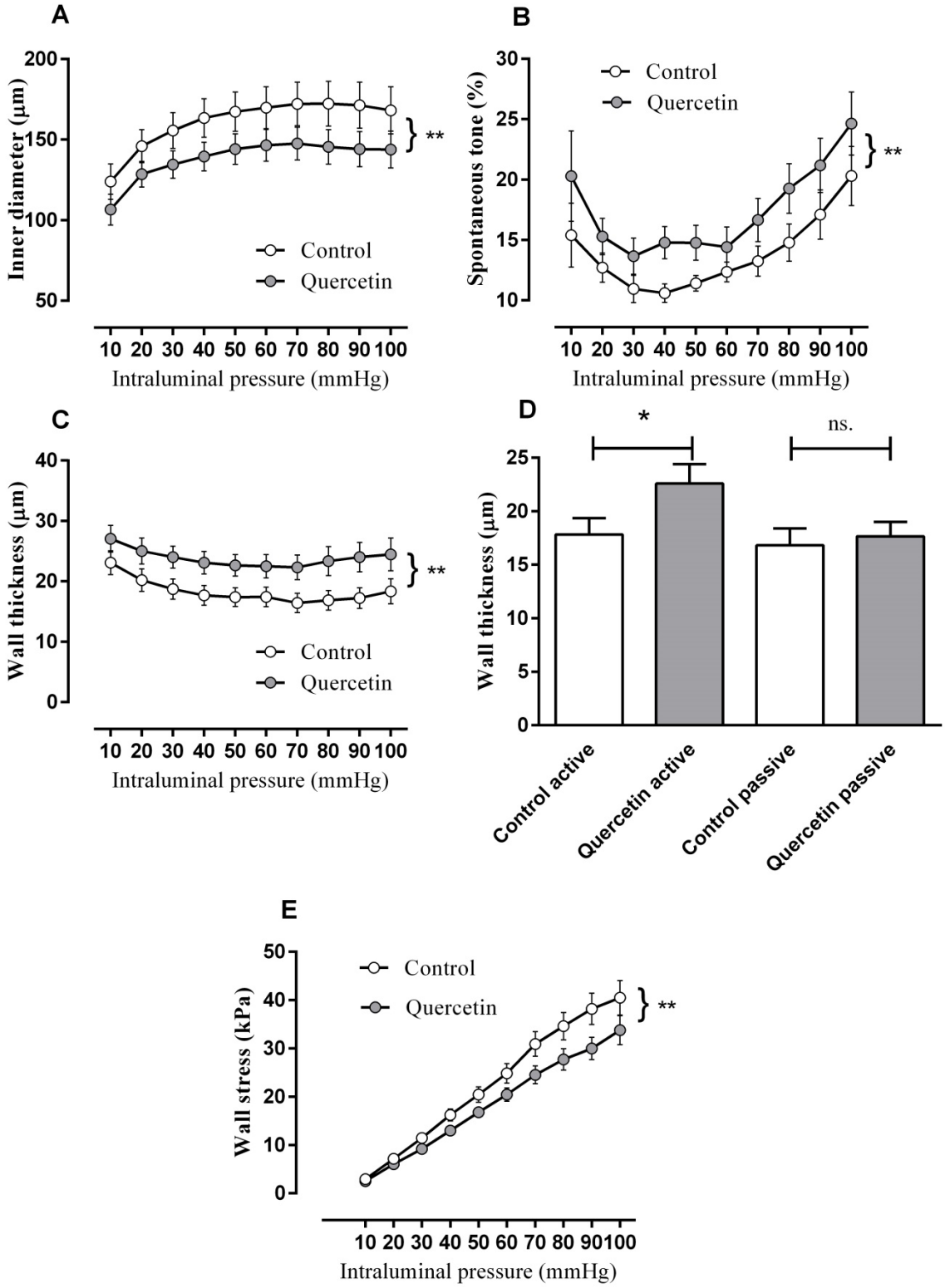
Figure 4. NO-mediated dilation. Panel A. Vasodilatation of spontaneously contracted segments induced by 10 μmol/liter acetylcholine. Note reduced acetylcholine dilation in the pressure range of 30-60 mmHg. (Two-way ANOVA $p<0.01$) Panel B. Vasodilatation of spontaneously contracted segments induced by 1 μmol/liter bradykinin. Note reduced bradykinin-stimulated dilation in the pressure range of 70-100 mmHg. (Two-way ANOVA $p<0.01$) Panel C. Additional vasoconstriction induced in spontaneously contracted segments by application of the NO synthase blocker L-NAME (100 μmol/liter). Note higher level of basal NO dilation of quercetin-treated segments. Significant between 60-100 mmHg intraluminal pressure. (Two-way ANOVA, $p<0.05$; n=10 quercetin treated, n=10 control, data from second series of experiment) Panel D.: Sum of basal and bradykinin-induced endothelial vasodilation. Basal NO-mediated dilatation is measured with application of L-NAME (bars with pattern). In control vessels this dilator effect is decreasing as a function of increasing intraluminal pressure, while in quercetin-treated vessels basal NO-mediated dilation is constant. Bradykinin induced endothelial vasodilation (bars without pattern) is a reserve of NO-mediated vasodilation. Note that maximum NO-induced vasodilation (sum of basal and induced NO-mediated dilation) did not differ between the two groups, whereas quercetin-treated segments showed higher basal NO dilation activity using up a higher portion of that maximum capacity under basal conditions.

1 Figure 1


A
Passive inner diameter (µm)
250
200
150
100
ns.
Control
Quercetin
10 20 30 40 50 60 70 80 90 100
Intraluminal pressure (mmHg)
B
E_{inc} (log(kPa))
3.0
2.5
2.0
1.5
ns.
Control
Quercetin
10 20 30 40 50 60 70 80 90
Intraluminal pressure (mmHg)

2

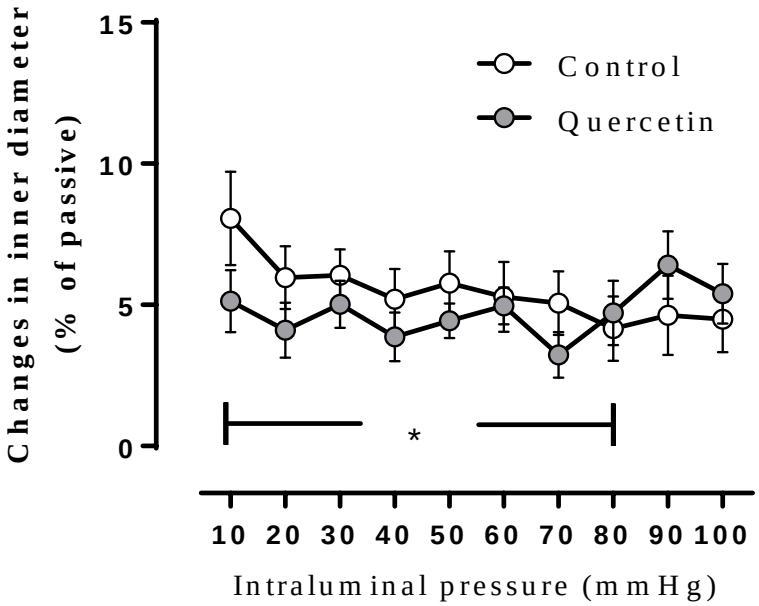
1 Figure 2


A
Inner diameter (µm)
200
150
100
50
Control
Quercetin
**
10 20 30 40 50 60 70 80 90 100
Intraluminal pressure (mmHg)
B
Spontaneous tone (%)
30
25
20
15
10
Control
Quercetin
**
10 20 30 40 50 60 70 80 90 100
Intraluminal pressure (mmHg)
C
Wall thickness (µm)
40
30
20
10
0
Control
Quercetin
**
10 20 30 40 50 60 70 80 90 100
Intraluminal pressure (mmHg)
D
Wall thickness (µm)
25
20
15
10
5
0
*
ns.
Control active
Quercetin active
Control passive
Quercetin passive
E
Wall stress (kPa)
50
40
30
20
10
0
Control
Quercetin
**
10 20 30 40 50 60 70 80 90100
Intraluminal pressure (mmHg)

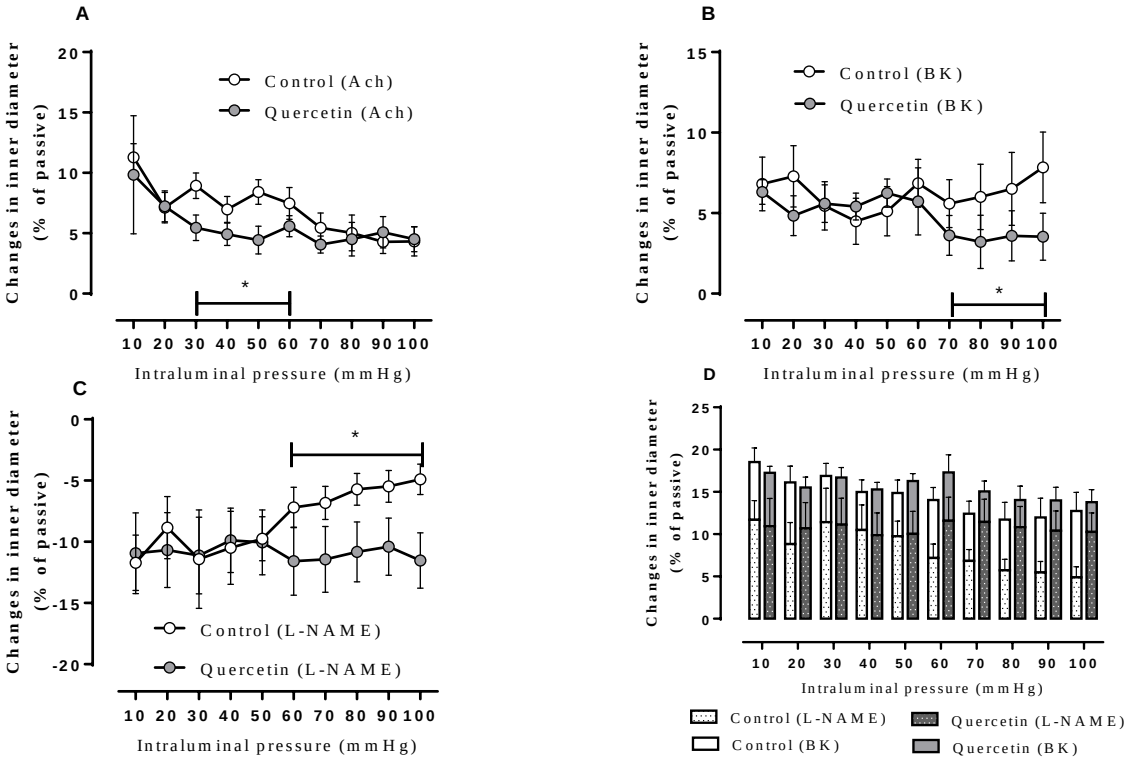
2

3

Figure 3


Changes in inner diameter
(% of passive)
15
10
5
0
Control
Quercetin
*
10 20 30 40 50 60 70 80 90 100
Intraluminal pressure (mmHg)

1 Figure 4
2



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4