Acute and chronic effects of the flavonoid quercetin on coronary arterioles

Ph.D. thesis

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Budapest
2018

1. Introduction

Cardiovascular disease morbidity and mortality is a global problem, and the leading cause of death in Hungary. The scientific community is looking after new therapeutic methods. Nobel prize winner Hungarian researcher, Albert Szent-Györgyi was interested in the beneficial properties of flavonoids on capillaries in 1936. The last 80 years provided us more and more details about the diverse flavonoids, including quercetin. However, there is a growing body of mechanistic studies about quercetin, just a few of them was published about its effect on coronary arteries, and almost no information is available regarding coronary arterioles. The utilization of this potent flavonoid requires detailed information about its vasodilator mechanism on the coronary arterioles. Therefore the topic of this thesis is the short-, and long-term effect of the flavonoid quercetin on the coronary arterioles.

Quercetin and its glycosylated forms, like rutin are frequent type of flavonoids. The daily intake is about 28-42 mg in case of a mixed diet, which leads to 0.1-1 μmol/liter plasma concentration.

Relatively few human studies were published about the acute effects of quercetin, but according to the literature it seems to increase microcirculatory flow by enhancing NO production at high doses (over 1000 mg polyphenol), but at lower doses (50-400 mg quercetin), its effect on the microcirculation is independent of NO. Based on animal experiments, quercetin has an endothel dependent vasodilation pathway, mediated by NO and vasodilatory prostanoids, and an endothel independent

way of action due to acting on L-type calcium channel and potassium activated calcium channel.
Long-term treatment with quercetin results in an improvement of the endothelial function, but the mechanism is not clear. In addition long-term quercetin treatment may lead to the remodeling of vessel structure and pharmacological responsiveness due to its antioxidant activity, interaction of many intracellular enzymes, inhibition of growth factor signal transmission and matrix metalloproteases.

2. Objectives

The vasodilatory action of quercetin is negatively correlated with anatomical lumen size: the vasodilation was more significant in small arteries than in large arteries. Is this tendency also present in even smaller arteries and arterioles?
Vasodilatory action of quercetin on coronary arteries is significantly endothel dependent, but there is no information about the ratio of NO and prostanoids in this vasodilation on coronary arterioles. Since NO has higher impact on small arteries, it can be assumed that another mediator plays the main role in arterioles.
After long-term quercetin supplementation, is there a change in basal NO mediated dilation? How do antioxidant and other mechanisms affect the age-induced biomechanical and pharmacological remodeling?
Based on these, we aimed to answer the following questions:

1. Is there a vasodilation in rat intramural coronary arterioles in response to physiologically relevant doses of quercetin? Is there is, what is the amplitude of vasodilatation?

2. Is the vasodilatory action of quercetin in these arterioles mediated by NO or by vasodilatory prostanoids?
3. How does the long-term quercetin supplementation influence the age-induced biomechanical and functional remodeling of coronary arterioles?

3. Methods

3.1. Animals

Rats were kept at the animal housing facility of Semmelweis University, Basic Medical Science Center under constant temperature and humidity, and animals received rat chow (S8106-S011 SM, Ssniff Spezialdiäten, Soest, Germany) and drinking water ad libitum. At the day of the experiment, rats were anesthetized with pentobarbital (i.p. 45 mg/kg), and the heart was removed and put into 4°C normal Krebs-Ringer solution (nKR, 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, EDTA 0.034). All procedures conformed to the national standards and guidelines of the European Union. IRB registration number is 22.0/2960/003/2009, with approval of the Institutional Animal Care and Use Committee of Semmelweis University at 09.28. 2009.

- *acute vascular effects of quercetin*

Male Wistar rats (350-450 g) were used. Animals acclimatized for 3 days prior the experiment.

- *long-term effects of quercetin supplementation*

Male Wistar rats (180-200 g) were divided into 2 groups: quercetin treated group received a supplementation of 30 mg/kg/day for 8 weeks via drinking water, and control group

was kept parallel. Animal behavior, body mass and turgor of the skin were checked twice a week.

3.2. Vascular preparation

Experiments were performed by pressure angiometry. 3 mm long segment of intramural coronary arterioles was prepared, cannulated at both ends and embedded into the organ bath (Experimetria LTD, Budapest, Hungary) at a stage of an inverted microscope. Optimal pH (~7.4) and partial oxygen tension (~95 mmHg) were maintained with bubbling (75% N_2, 20% O_2, 5% CO_2). Servo controlled pumps were connected to both cannulas, in order to maintain 50 mmHg intraluminal pressure during incubation, and to change it between 10-100 mmHg in a stepwise manner during pressure gradient protocol with 3 min incubation at each step. All experiments were performed under no flow condition. Vascular diameter was measured off line (Leica QWin, Wetzlar, Germany) after calibration with micrometer etalon (Wild, Heelbrugg, Switzerland)

3.3. Vascular protocols

- *Dose response relationship of acute quercetin effect*

These experiments were performed on coronary arterioles with anatomical lumen size of 151±27 μm (measured in calcium free Krebs Ringer solution, Cafree, 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, EDTA 0.025, n=8). After initial incubation in nKR, quercetin concentration was elevated from 10^{-9} mol/liter to 10^{-5} mol/liter. Reproducibility was checked with repeated incubation in nKR, and the maximal, anatomical diameter was determined in Cafree solution.

- *Mechanism of endothelial vasodilatory action of quercetin*

This study was performed on coronary arterioles with 100-320 μm (188±14 μm) anatomical lumen diameter (n=16). After initial incubation in nKR, vasodilatory mechanism was examined with COX blocker indomethacin and eNOS blocker L-NAME in presence of 10^{-7} mol/liter quercetin. Reproducibility was tested with repeated incubation in nKR and 10^{-7} mol/liter quercetin then anatomical lumen size was determined in Cafree.

-Effect of long-term supplementation on biomechanical and pharmacological remodeling

The anatomical lumen diameter of arterioles in this study was 174±4 μm (n=22 quercetin treated and n=20 control). Pharmacological remodeling was tested with 10^{-5} mol/liter norepinephrine, 10^{-5} mol/liter acetylcholine, 10^{-6} mol/liter bradykinin, and 10^{-4} mol/liter L-NAME. Reproducibility was checked with repeated nKR incubation. Biomechanical remodeling was examined with pressure gradient in Cafree solution.

3.4. Calculations

Data was normalized to anatomical diameter at the same intraluminal pressure. Spontaneous tone and active tone were calculated as follows: 100- normalized diameter. Tangential was stress was calculated based on the Laplace-Frank equation, and incremental elastic moduli was calculated based on the formula of Cox 1979.

3.5. Statistics

Normal distribution of the data was checked with Shapiro-Wilk test. Normally distributed data is presented as mean±SEM, and was analyzed with one-way, two-way ordinal or repeated

measures ANOVA with Tukey post hoc-test, t-test, Pearson correlation and linear regression. Data with not normal distribution is presented as median and IQR [25%; 75%], and was analyzed with Mann-Whitney test. Statistical significance was considered if $p<0.05$.

4. Results

4.1. Dose-response relationship of quercetin on intramural coronary arterioles

Original spontaneous tone of coronary arterioles (20.2±4.3%) was significantly decreased after application of 10^{-9} mol/liter quercetin concentration. Near to physiological plasma concentrations (10^{-7} mol/liter) the active tone decreased to its half, while 10^{-5} mol/liter quercetin completely relaxed the coronary arterioles. This relaxation was reversible, and original tone returned after washing out (one-way repeated measures ANOVA, Tukey post-hoc test, $p<0.05$ vs. nKR at all concentrations).

4.2. Mechanism of endothelial vasodilatory action of quercetin

10^{-7} mol/liter quercetin significantly relaxed the coronary arterioles, and reduced their tone (21.3±2.3%) to the half (48.9±6.7) (two-way repeated measures ANOVA, Tukey post hoc-test 10-70 mmHg, $p<0.05$ vs. nKR). Amplitude of relaxation can be compared after normalization to the spontaneous tone of the arteriole (as precontraction). This normalized effect does not correlate with anatomical lumen diameter (Pearson correlation, r=-0.03, ns.). Nevertheless smaller arterioles have higher spontaneous tone, therefore *in vivo* the relaxation of smaller arterioles is more significant than

anatomically wider arterioles. Both indomethacin and L-NAME caused vasoconstriction of arterioles pretreated with quercetin (indomethacin: two-way repeated measures ANOVA, Tukey post hoc-test 20-60 mmHg, $p<0.05$ quercetin+ indomethacin vs. quercetin, L-NAME: two-way repeated measures ANOVA Tukey post hoc-test 10-70 mmHg $p<0.05$ quercetin+indomethacin+L-NAME vs. quercetin+indometacin). This suggests that vasodilation is mediated by NO and vasodilatory prostanoids. Comparison of amplitude of vasoconstriction at 50 mmHg shows higher vasoconstriction amplitude of indomethacin than L-NAME (7.8% [3.1; 10.5] vs. 3.5% [1.3; 5.1], Mann Whitney test $p<0.01$). Vasoconstriction caused by indomethacin is negatively correlated with anatomical lumen diameter (Pearson correlation, $r=-0.65$, $p<0.01$).

4.3. Effect of long-term supplementation on biomechanical and pharmacological remodeling

At the end of the 8 week long treatment, neither body weight (507±9 g vs. 504±10 g, t-test, ns.) nor the heart weight (1.38±0.14 g vs. 1.35±0.18 g, t-test, ns.) differed between the rats of the control and quercetin treated groups.

Control and quercetin treated arterioles did not differ in anatomical lumen size or elastic moduli (both comparisons: two-way ANOVA, ns.). However, spontaneous tone of quercetin treated arterioles was higher (two-way ANOVA, $p<0.01$). This leads to significantly decreased lumen diameter under active conditions (nKR solution, two-way ANOVA, $p<0.01$). Vascular wall thickness of fully relaxed arteries did not differ (Mann Whitney test, ns.), but as a consequence of

higher tone, under active conditions, quercetin treated arterioles had thicker vascular wall (Mann Whitney test, $p<0.05$). This alteration leads to about 30% decrease in tangential wall stress in the quercetin treated group (two-way ANOVA, $p<0.01$). In case of biomechanical remodeling the key element is the higher spontaneous tone, which caused thicker vascular wall, decreased wall stress and significantly higher dilatory reserve.

Examining the pharmacological remodeling, we found decreased vasodilatory action of norepinephrine (two-way ANOVA, $p<0.01$), which can be explained by the inhibitory action of quercetin and its metabolites to adenylate cyclase according to the literature. Both endothel dependent vasodilator (acetylcholine and bradykinin) caused decreased vasodilatation in treated arterioles (both comparisons: two-way ANOVA, $p<0.01$), but L-NAME induced vasoconstriction was higher in quercetin treated arterioles (two-way ANOVA Tukey post hoc-test 60-100 mmHg, $p<0.05$). Summarizing the observed basal NO dependent vasodilatation and maximal NO dependent vasodilatation it is obvious that the range of total NO dependent dilatation did not differ between the two groups (two-way ANOVA, ns.). However, basal activity of eNOS was higher in the quercetin treated group (comparing linear regression lines, $p<0.05$), especially at higher intraluminal pressures, when basal NO dependent vasodilatation decreased in control arterioles, but remain at the same level in quercetin treated arterioles.

5. Conclusions

1. Is there a vasodilation in rat intramural coronary arterioles in response to physiologically relevant doses of quercetin? If there is, what is the amplitude of the vasodilatation?

1 nmol/liter quercetin concentration dilated significantly the intramural coronary arterioles. The amplitude of the relaxation increases in a dose dependent manner until 10 μmol/liter, which almost fully relaxed the coronary arterioles. Average plasma concentration of 100 nmol/liter causes significant and physiologically meaningful vasodilatation, which is about 10% of the anatomical diameter, while reduces the spontaneous tone to the half. Coronary arterioles with smaller anatomical size have higher spontaneous tone, therefore vasodilatation of these arterioles are more significant.

2. Is the vasodilatory action of quercetin in these arterioles mediated by NO or by vasodilatory prostanoids?

Our results show that the vasodilatory action of quercetin on intramural coronary arterioles is mediated by both NO and vasodilatory prostanoids. Based on anatomical lumen size prostanoid mediated dilatation may be dominant and more important in smaller arterioles (under 150-180 μm).

3. How does the long-term quercetin supplementation influence the age-induced biomechanical and functional remodeling of coronary arterioles?

Two month supplementation with quercetin at 30 mg/kg/day dose increased the spontaneous tone and basal NO mediated dilatation of coronary arterioles. This result in increased the vascular wall thickness, thus decreased the isobar wall stress

and increased the vasodilatory reserve to the coronary arterioles.

6. Bibliography of the candidate's publications

Publications related to the thesis:

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