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1 # Cystic fibrosis transmembrane conductance regulator is involved in
2 polyphenol-induced swelling of the endothelial glycocalyx

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7 **Abstract** Q4

8 Previous studies show that polyphenol-rich compounds can induce a swelling of the endothelial glycocalyx (eGC). Our goal was to reveal
9 the mechanism behind the eGC-swelling. As polyphenols are potent modulators of fibrosis transmembrane conductance regulator (CFTR)
10 Cl^- channel, the hypothesis was tested whether a polyphenol-induced increase in CFTR activity is responsible for the eGC-swelling. The
11 impact of the polyphenols resveratrol, (−)-epicatechin, and quercetin on nanomechanics of living endothelial GM7373 cells was monitored
12 by AFM-nanoindentation. The tested polyphenols lead to eGC-swelling with a simultaneous decrease in cortical stiffness. EGC-swelling, but
13 not the change in cortical stiffness, was prevented by the inhibition of CFTR. Polyphenol-induced eGC-swelling could be mimicked by
14 cytochalasin D, an actin-depolymerizing agent. Thus, in the vascular endothelium, polyphenols induce eGC-swelling by softening cortical
15 actin and activating CFTR. Our findings imply that CFTR plays an important role in the maintenance of vascular homeostasis and may
16 explain the vasoprotective properties of polyphenols.
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18 *Key words:* Resveratrol; Quercetin; Epicatechin; Cortical actin; Atomic force microscopy; Cell cortex

19
Q5 ## Introduction

21 The luminal side of the endothelial cells is lined by a viscous
22 liquid surface layer, rich in carbohydrates, the endothelial
23 glycocalyx (eGC). The prominent constituents of the eGC are
24 the proteoglycans, formed by a core protein and one or more
25 attached carbohydrate side chains (glycosaminoglycans; GAGs).
26 Most commonly GAG side chains are composed of heparan sulfate
27 (50-90%), the rest is formed by hyaluronic acid and chondroitin,
28 dermatan, and keratin sulfates.[1] Some types of core proteins are
29 firmly attached to the cell surface (glypicans through a
30 glycosylphosphatidylinositol anchor and syndecans are transmem-
31 brane core proteins), whereas other proteoglycans, such as
32 decorins, versicans, perlecans, biglycans, and mimecans, were
33 secreted. The eGC is not a static structure but, under physiological
34 conditions, in a dynamic equilibrium. Enzymatic degradation,
35 shear-induced shedding, variable surface expression and biosyn-
36 thesis of proteoglycans/glycoproteins as well as variations in GAG
37 side chain sulfation lead to ongoing changes in the composition,
38 thickness and functional properties of the eGC.[1,2] The proteogly-
39 cans together with plasma proteins, lipids, and ions form a dynamic
40 and complex interface between blood and tissue with specific
41 functions, mainly the protection of the endothelium and the
42 regulation of vascular permeability.[3] The eGC binds oxygen-
43 scavenging enzymes like the extracellular superoxide dismutase,
44 reducing oxidative stress and preventing endothelial dysfunction.[1]
45 The eGC controls the cell surface microenvironment, thus
46 representing a regulatory domain for biochemical processes on
47 the cell surface.[1] It acts as a molecular sieve and a sensor of fluid
48 shear stress.[1,4,5] Furthermore, eGC affects blood rheology by
49 reducing flow resistance due to its negative charge. The eGC forms
50 a cell free layer by repelling red blood cells, which are also covered
51 with a negatively charged glycocalyx,[6] mitigating the wall shear

Conflicts of interest: None.

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stress.[7] Under physiological conditions adhesion molecules (VCAMs, ICAMs and PECAM) are concealed by the eGC preventing the attachment of leukocytes to the blood vessel wall.[8,9]

Due to the broad spectrum of functions, it is obvious that an intact eGC is a prerequisite for a healthy vasculature. Damage of the eGC can lead to vascular dysfunction like edema formation, high blood pressure, accelerated inflammation, atherosclerosis, platelet aggregation, hypercoagulability and loss of vascular responsiveness.[2,10-15] Thus, an understanding of eGC function and regulation is essential for the prevention and treatment of these vasculopathies.

Recently, it was shown that WS 1442, an ethanolic extract from *Crataegus* spp. induces an increase in the eGC volume and a decrease in eGC stiffness, a process called "eGC swelling". The eGC swelling is accompanied by an alteration of eGC function.[16] Since the understanding of eGC regulation could be the key for an appropriate treatment of different vasculopathies, the present study aims to disclose the mechanisms behind the WS 1442-induced eGC swelling. WS 1442 is rich in polyphenols, which are known for their vasoprotective effects.[17,18] Some polyphenols (e.g. quercetin, resveratrol) are capable of activating the cystic fibrosis transmembrane conductance regulator (CFTR).[19-22] In airway epithelia, CFTR regulates, among others, the height of the PCL (periciliary layer) by maintaining the ion and water homeostasis. This keeps the glycocalyx highly hydrated and preserves the low viscosity of the PCL.[23] CFTR is not only a chloride channel, but also a transporter of bicarbonate (HCO_3^-).[24] Before secretion, proteoglycans (constituents of the glycocalyx, and mucins) are highly ordered in vesicles at high calcium and low pH. After release, calcium is removed and pH increased due to bicarbonate, thus allowing the polymeric proteoglycans to expand to their highly hydrated gel-like structure.[25] Therefore, HCO_3^- transport, and hence CFTR are suspected to play a key role in the epithelial glycocalyx formation. However, there is evidence that CFTR is not only expressed in epithelia, but has been described also in the vascular endothelium.[26-28]

In this work, we tested the hypothesis that polyphenols act on eGC through the activation of CFTR. We tested (i) the impact of resveratrol, (−)-epicatechin and quercetin on the eGC stiffness *k(eGC)* and height *dx(eGC)* and (ii) the role of CFTR in polyphenol-induced eGC swelling, using CFTR inhibitors. By applying nanoindentation methods we found that polyphenols soften the cortical actin web of endothelial cells paralleled by CFTR activation and eGC swelling.

Methods

Reagents

All chemicals were purchased from Sigma Aldrich (Steinheim, Germany) unless stated otherwise.

Cell culture

For experiments, bovine aortic endothelial GM7373 cells (DSMZ, Braunschweig, Germany) in the passages 6-20 were used. The cells were cultured at 37 °C and 5% CO_2 in minimal essential medium (Invitrogen Corp., La Jolla, CA, USA) supplemented with 20% fetal calf serum (FCS; PAA Clone, Coelbe, Germany), 1% MEM vitamins (Invitrogen), 1% MEM nonessential amino acids (Invitrogen) and 1% Pen-Strep solution (10,000 U/ml penicillin, 10 μg/ml streptomycin; Invitrogen). As positive control in Western blot and immunofluorescence experiments, human bronchial epithelial 16HBE14o- cells were used. The cells were cultured at 37 °C and 5% CO_2 in minimal essential medium with L-glutamine (PAA Clone) supplemented with 10% FCS, 1% MEM nonessential amino acids (Invitrogen), 1% Pen-Strep solution (10,000 U/ml penicillin, 10 μg/ml streptomycin; Invitrogen). For atomic force microscopy (AFM) and immunofluorescence experiments, the cells were seeded on Ø15 mm glass cover slips and used after forming a confluent monolayer. For Western blot, the cells were cultured in Ø100 mm culture dishes until confluence was reached.

Atomic force microscopy

The nanomechanical properties of the cells were studied by force-indentation measurements as described previously.[16] Briefly, it could be shown that endothelial cells consist of different functional layers with distinguishable stiffness. We could show that the endothelial glycocalyx is a very soft structure, thus deformation of the eGC is mainly reflected by the first part of the force distance curve. Enzymatic digestion of the glycocalyx constituents revealed that the first part of a force distance curve is evoked by the eGC as the length and slope of this part of the force distance curve were reduced after enzymatic treatment.[16,29,30] No changes in the second part of the force distance curves could be observed.[29] Additionally, it could be shown that the slope of the first part of force distance curve, obtained on endothelial cells, strongly correlates with the amount of eGC-constituents.[16,30] A MultiMode AFM (Bruker, Karlsruhe, Germany) was used. The cells were indented with a soft cantilever (spring constant = 11 pN/nm; Novascan, Ames, IA, USA) equipped with a spherical polystyrene tip (Ø = 1 μm). An indentation velocity of 300 nm/s and a maximal loading force of 300 pN were applied. Force–indentation curves were obtained, from which the stiffness *k* and thickness *dx* of different cellular layers were derived (Figure 1). We used stiffness (Newton per meter) instead of elasticity (Young's modulus) in order to avoid using assumptions that are not applicable for glycocalyx. Even the more sophisticated "brush model" developed by Sokolov and coworkers[31,32] is not applicable since the latter describes the contribution of the pericellular brush (various membrane protrusions and corrugations like microvilli, microridges, filopodia and glycocalyx) to cells' mechanics rather than the glycocalyx as such. The analysis of the force–indentation curves was performed with Punias software (Release 1.8; http://punias.voila.net/). Determination of the contact point was done by linear interpolation of the non-contact region (baseline fit). The deviation of the curve from this baseline fit was identified as contact point. Starting from the contact point a linear fit was performed within the apparent linear regime of the curve. The goodness of linear fits is displayed in PUNIAS by showing the coefficient of determination R^2 during fit range determination in real-time. The algorithm allows delimiting the fit range by defining the minimal R^2 value. In this work the boundary condition was set to $R^2 > 0.8$. AFM measurements were performed in HEPES buffered solution (standard composition in

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