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The enigma of flavonoids and bilitranslocase activity in the cardiovascular system

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Abstract — Numerous epidemiologic studies showed an inverse correlation between dietary flavonoid consumption and cardiovascular risk, but the exact mechanisms are still largely unknown. Flavonoids exhibit hormetic properties, where low concentrations activate adaptive cellular stress response pathways and thus lead towards cytoprotection, whereas high concentrations are cytotoxic. However, the limited bioavailability of dietary flavonoids doubts the relevance of effective flavonoid intracellular concentrations to induce bioactivity in endothelial cells. Therefore, translocation of flavonoids through the cell plasma membrane must occur via specific transporter proteins. Hereby, we describe the involvement of the membrane transporter bilitranslocase (TC #2.A.65.1.1) as the key underlying molecular mechanism for membrane transport, which might help resolve the enigma of flavonoids bioactivity.

Index Terms — bilitranslocase, flavonoids, cardiovascular health, hormesis, TRANS2CARE

1 FLAVONOIDS AND CARDIOVASCULAR HEALTH

Numerous epidemiological studies have shown that regular intake of flavonoids is associated with an improved cardiovascular prognosis [1,2]. In the setting of primary prevention, individuals with the highest flavonoid intake have modestly reduced risks for developing cardiovascular disease [3-5]. In addition, flavonoid intake benefits individuals with established cardiovascular disease. This can be seen from clinical signs like lowered blood pressure [6-8], improved endothelial function [9-11], inhibited platelet aggregation [7,12-14], decreased low density lipoprotein oxidation [15,16], and reduced inflammatory responses [17-19].

To promote the idea of healthy diet, it is now clear that the medical researcher must put big efforts in providing concise explanations how the potential beneficial effects of flavonoids can arise. Scientists have a responsibility to investigate this issue, and provide the general public evidence-based guidelines on a healthy diet. Even more, researchers must try to isolate the therapeutic lead molecules, and chemically improve them, to obtain new potential drugs for treating specific pathophysiological conditions. So far, exact mechanisms of how flavonoids act are still largely obscure, and thus represent a big challenge for research, whether in academia or in the pharmaceutical and the nutraceutical industry.

2 PHARMACOKINETIC CONCERNS RELATED TO THE FLAVONOIDS PHARMACODYNAMICS

The biggest pharmacological concern of dietary flavonoids can be attributed to their limited bioavailability, which results in very low plasma concentrations (0.1-1 μM) [20]. This questions whether intra-cellular concentrations attain levels that are of any relevance to the endothelial function [21]. In fact, it is not clear if flavonoids interact with any endothelial plasma membrane receptors and thus act by triggering an intracellular signal transduction cascade, or they penetrate into the cells and, consequently, bind to intracellular molecular targets. In the latter hypothetic pathway, translocation of flavonoids through the cell plasma membrane must occur via specific transport proteins [22].

On the other hand, high plasma flavonoid concentrations might have deleterious systemic effects, as it is suggested by some of theirs on the cardiovascular system [23]. Though still isolated, these observations warn against the potential alternative routes of administration, i.e. parenteral, which would avoid the low absorption and first-pass metabolism issues, or utilization of the enhanced absorption delivery systems.

It is widely accepted that flavonoids are good antioxidants when chemically tested in in vitro non-biological systems, but little emphasis is put on the fact that most of those studies showed effects in the supra-physiological concentration ranges, many magnitudes of order higher than reported in vivo. Thus, there is now a more plausible hypothesis that the in vivo beneficial effects of these phytochemicals are unlikely to be explained just by their per se antioxidant capability. Flavonoids may exhibit hormetic properties, by acting as `low-dose stressors' that may prepare cells to resist more severe oxidative stress, while high concentrations are cytotoxic [24]. In this biphasic manner, low concentrations of flavonoids induce cardiovascular protection, while high concentrations are cardiotoxic [23].

This is meaningful since low and transient concentrations of flavonoids in the plasma contribute little per se to the overall plasma antioxidant status, when compared to the relatively higher concentrations of other antioxidants in plasma, e.g. vitamin C, uric acid, bilirubin, etc. Taken altogether, the diverse chemical structures of flavonoids exhibit molecular mimicry with many mammalian endogenous molecules, thereby leading to manifold biological effects, i.e. altering intracellular signaling pathways and

enzyme activities. Compared to the specific activity of other plant-related chemicals, for example morphine's high affinity targeting of the opiate receptors, flavonoids have been always on the borderline between pharmaceuticals and xenobiotics. Since they don't have any specific cellular targets, as indirectly confirmed by all flavonoids-related studies with only partial inhibition and/or activation of numerous examined molecular targets, they should, in our opinion, fall in the category of xenobiotics.
From an evolutionary perspective, flavonoids are primarily produced by plants to prevent insects and other animals to eat plants, and this again speaks in favor that the observed bioactivity of flavonoids from animal and human studies is actually resulting from the activation of adaptive cellular stress response pathways [25,26]. On the other hand, we must also consider the plausibility that there do exist some conservative proteins in nearly all animals, who eat the flavonoid-rich plants, and there are also similar proteins found in both the plant and animal kingdoms. Many of these are still largely unknown in humans, but can profoundly influence both the pharmacokinetic or pharmacodynamics properties of flavonoids.

3 BILITRANSLOCASE TRANSPORTS FLAVONOIDS INTO THE CELLS

There is a big question in the science: how can flavonoids enter into the cells? A potentially involved carrier is bilitranslocase (TC #2.A.65.1.1) [27], a bilirubin-specific membrane protein that is also responsible for ATP-independent transport of flavonoids across cell membranes in various rat organs, e.g. on the epithelium of liver, gastrointestinal system, kidney, brain, lungs, etc., and also on the vascular endothelium [28]. Even though the investigation has not yet covered all mammalian tissues or other species, data already suggest that bilitranslocase might be a ubiquitous membrane protein, therefore playing a major role in biology.
Based on extensive transport activity assays performed on a battery of flavonoids subclasses, bilitranslocase can be considered as an anthocyanin-selective transporter, since other flavonoids are not effective ligands [27]. This selective interaction can be explained in terms of molecular mimicry between bilirubin and anthocyanins, as well as by specific structural characteristics of anthocyanins, such as the planar system of conjugated aromatic rings and pH-dependent tautomerism, which by protonation converts the quinoidal form into the phenolic form [29].
Bilitranslocase is expressed both in the vascular system, on the endothelium and vascular smooth muscle cells [28], and also in the cardiac system, on the endocardium and cardiomyocytes (data not yet published). This presence enables to understand some of the cardiovascular effects of flavonoids, which are summarized in the Table 1.

1. DIMINUTION OF OXIDATIVE STRESS	Direct scavenging of free radicals (antioxidant action in the narrow sense of the word)
	Metal interaction (iron and/or copper chelation)
	Inhibition of ROS producing enzymes, in particular xanthine oxidase, NADPH oxidase and lipoxygenases
	Stimulation of endogenous antioxidant defense mechanisms, increase in expression of eNOS, intracellular glutathione
2. DECREASE IN THE EXPRESSION OF INFLAMMATORY SIGNALING MOLECULES	Inhibition of iNOS expression (endothelium)
	Inhibition of COX-2 expression (endothelium)
	Inhibition of leukocyte activation
3. INHIBITION OF PLATELET AGGREGATION	Increase in platelet NO production
	Decrease in platelet production of superoxide anion
4. DIRECT VASODILATORY ACTION	Activation of eNOS signaling pathway in the endothelium, leading to release of NO
	Activation of EDHF-mediated vasorelaxation in the endothelium
	Direct effects on ion channels
	Inhibition of cyclic adenosine monophosphate-dependent phosphodiesterase
5. OTHER CARDIOVASCULAR ACTIVITIES	Isoflavones improve endothelial function through an effect on αestrogen receptor
	Positive/negative inotropic effects
	Activation of PPARγ

Table 1. List of potential protective effects of flavonoids in the cardiovascular system. Adapted from [30].

4 ROLE OF BILITRANSLOCASE IN THE CARDIOVASCULAR SYSTEM

We have made a significant progress in our understanding of the functional role of the bilitranslocase protein in the cardiovascular system. Original approach towards studying the protein function was based upon the inhibition of the bilitranslocase activity by using anti-sequence bilitranslocase antibodies. They target distinct extracellular epitopes of the carrier, and thereby inhibit the transport of the substrates into the cytoplasm.

The data obtained from our experiments suggest that even low concentrations (in nM range) of flavonoids, which are comparable to post-absorption plasma levels, are rapidly (<1min) taken up into the endothelium via bilitranslocase (Ziberna, FRBM, under review). This rapid uptake into endothelial cells can be explained only by the

involvement of specific membrane transporters that catalyze the passage of polar substances, such as flavonoids, through the phospholipid bilayer.
Furthermore, bilitranslocase represents a key step in the flavonoids-induced endothelial cell-signaling cascade leading to the activation of intracellular PI3-kinase/Akt/eNOS pathway (Ziberna, not yet published), as schematically shown on Figure 1. Accordingly, bilitranslocase mediates the vasorelaxation responses induced by flavonoids [31].

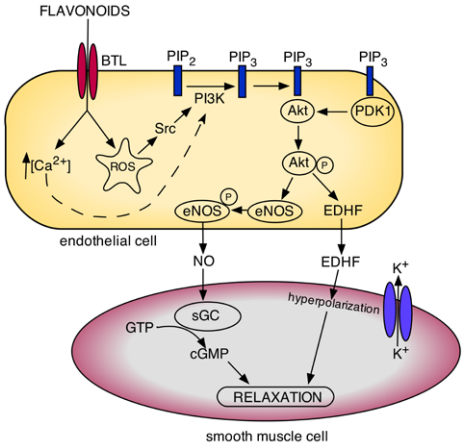

Figure 1. Scheme indicating that flavonoids are potent inducers of the endothelial formation of NO and EDHF with their corresponding different intracellular signaling pathways. BTL – bilitranslocase, EDHF – endothelium derived hyperpolarizing factor, NO – nitric oxide, eNOS – endothelial NO synthase, PI3K – phosphatidyl-inositol 3-kinase, PDK1 – posphoinositide-dependent kinase 1, ROS – reactive oxygen species, sGC – soluble guanylyl cyclase. Adapted from [30].

In the cardiac system, we introduced an innovative approach of retrograde coronary perfusion with the bolus of the bilitranslocase antibodies. Thus, specific immunocomplexes were formed on the surface of the coronary endothelium and in a lesser extent, if at all, on the endocardium of the left atrium and ventricle. Importantly, unbound immunoglobulins were removed by continuous perfusion. We found that bilitranslocase mediates the cardioprotective activity of flavonoids in ischemia-reperfusion model of the isolated heart, as observed by measuring coronary flow, cardiomyocyte lysis and duration of arrhythmias (Ziberna, FRBM, under review).

5 INTERPLAY BETWEEN FLAVONOIDS AND BILIRUBIN

Given the mutual metabolic interference between flavonoids and bilirubin, it can be speculated that the transient increase in the antioxidant capacity of endothelium following ingestion of flavonoids [32] might be, in part, ascribed to a transient and reversible decrease of bilirubin clearance by the endothelial cells. Furthermore, it can be speculated that flavonoids occupy the activity of bilitranslocase, thus decreasing the endothelial efflux of intracellular (endogenous) bilirubin, which is constantly produced by heme oxygenase-1 and biliverdin reductase. Since bilirubin is a strong

endogenous antioxidant, it can be speculated that even a very small increase in basal levels of free bilirubin can provide significant antioxidant protection of endothelium [33]. In fact, patients with Gilbert's syndrome, a hereditary condition characterized by slightly elevated bilirubin plasma levels and occasional mild and intermittent jaundice, show a decreased risk of cardiovascular diseases in comparison to normo-bilirubinemic populations [34,35].

6 CARDIOVASCULAR RESEARCH ON THE BILITRANSLOCASE WITHIN TRANS2CARE PROJECT

In the framework of TRANS2CARE project, we will continue to further elaborate our cardiovascular research on the role of bilitranslocase, jointly with our project partners:

- collaboration with PP1 – Chemical Institute of Ljubljana: in the field of computational modeling and chemometrics (QSAR);
- collaboration with PP3 – University of Nova Gorica: studying the metabolism and fast-uptake of flavonoids and bilirubin into endothelial cells;
- collaboration with PP10 – Blood Transfusion Center of Slovenia: development of novel monoclonal antibodies against bilitranslocase, which are crucial for our platform of functional studies, as well as having the potential to be used as biomarkers;

Furthermore, we will also expand our network, in accordance with WP7 - Enlargement and consolidation of the network, towards the research groups outside the network:

- collaboration with University of Trieste, Department of Materials and Natural Resources, CENMAT, contact person Alois Bonifacio: employing SERS Raman spectroscopy to detect low concentration of bilirubin in the extracellular and intracellular environment;
- collaboration with IASMA Research and Innovation Center, Food Quality and Nutrition Area, San Michele all'Adige, contact person Fulvio Mattivi: metabolomics experiments on the effects of flavonoids in cardiovascular tissues.

7 CONCLUSION

The knowledge presented is important for the better understanding of diet-based preventive medicine, which is an affordable and efficient way of improving worldwide health. Academic world must, therefore, provide the rationale for specific dietary guidelines about including flavonoids-rich food in the diet. And the people will have no hesitation to make excellent life choices in the direction of achieving significant health benefits.

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