Perspective

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The flavonoid paradox: conjugation and deconjugation as key steps for the biological activity of flavonoids

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Abstract

Flavonoids have been proposed to exert beneficial effects in the prevention of a large number of diseases, including cancer, cardiovascular disease, and neurodegenerative disorders. Paradoxically, despite the most representative flavonoid – quercetin – exerting biologically demonstrable systemic effects, it is not found in plasma after oral administration and its circulating metabolites show weak activity *in vitro*. The current available evidence indicates that quercetin is extensively metabolized into methylated and glucurono- and sulfo-conjugated metabolites, which are the plasma circulating forms; and glucurono-, but not sulfo-conjugates, can be hydrolyzed at the vascular level, yielding the parent aglycone which accumulates in tissues. Thus conjugation is a reversible process and, at least regarding the vasodilator and antihypertensive effects, the conjugation-deconjugation cycle appears to be an absolute requirement. Glucuronidated derivatives transport quercetin and its methylated form, and deliver to the tissues the free aglycone, which is the final effector.

Keywords: quercetin; glucuronide; metabolism; vasodilation; hypertension.

In vitro cognitio sed in vivo veritas.
(Richard Gryglewski)

Flavonoids have been proposed to exert beneficial effects in the prevention of a large number of diseases, including cancer, cardiovascular disease and neurodegenerative disorders. We have recently reviewed the continuously growing evidence supporting a beneficial role of flavonols on cardiovascular disease and the potential molecular targets involved.[1] More than 35 000 studies have been published on flavonoids and nearly 10 000 with just a single one of them: quercetin. However, some fundamental questions regarding efficacy, mechanism of action and bioavailability of flavonoids are still unanswered.

Flavonoids are a class of plant phenolic compounds present in fruits, vegetables, nuts, spices, herbs and derived products such as wine, tea and chocolate. The flavonoid class includes several thousand compounds as found in nature and comprises several subclasses such as flavonols, flavones, flavanones, flavanols, anthocyanidins, isoflavones, dihydroflavonols and chalcones. They are regularly ingested in the diet as complex mixtures of different flavonoid compounds together with other active substances. After oral ingestion, flavonoids are subject to chemical modification within the gastrointestinal tract by the enzymes of the host and microbiota and further metabolized after absorption in the intestinal wall, the liver and peripheral tissues.[2] The food matrix may also influence the gastrointestinal metabolism and absorption. Thus, within our regular daily diet, a multitude of compounds are present in the gastrointestinal lumen, blood and tissues. The concentrations achieved for most of these substances are below the micromolar range and remain undetected by common analytical techniques, and only a small number of compounds capture the attention of scientists. However, low concentrations may not necessarily indicate lack of biological activity for a highly potent substance. Herein we will focus on quercetin, one of the most widely distributed flavonoids in the diet.

The early studies on pharmacokinetics of quercetin in humans[3] found that oral absorption of quercetin was minimal and no measurable concentrations in plasma or urine could be detected and, therefore, the authors concluded that oral administration of flavonoids may be of questionable value. Later studies confirmed that quercetin as an aglycone was undetectable in plasma.[4] Paradoxically, a large number of human and animal studies show that oral administration of quercetin exerts a clear systemic biological effect.[1,5] Therefore, the flavonoid paradox can be stated as follows: *Despite quercetin not being found in plasma after oral administration, it exerts biologically demonstrable systemic effects.*

It has become clear during the last decade that despite quercetin being absent in plasma as an aglycone, it is indeed present

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conjugated with glucuronic acid and sulfate. Quercetin may be administered orally as an aglycone in food supplements or ingested as glycosylated forms in the diet. Glycosides are hydrolyzed by the mouth and intestinal bacteria and by the lactase phloridizin hydrolase of the brush border of epithelial cells, but some may be also absorbed intact via the glucose transporter SGLT1 and further hydrolyzed intracellularly. Quercetin is rapidly conjugated with glucuronic acid and/or sulfate during first-pass metabolism in the intestinal wall or in the liver, and a portion of the metabolites is also methylated. The enzymes responsible for these metabolic processes are UDP-glucuronosyltransferases, sulfotransferases and methyltransferases and the major metabolites of quercetin in human plasma[4] are quercetin-3-glucuronide, quercetin-3′-sulfate and isorhamnetin-3-glucuronide (3′-methyl-quercetin-3-glucuronide).

A huge amount of scientific data has been generated over decades with quercetin aglycone and other related flavonoids *in vitro*. Because quercetin cannot be found in plasma, the relevance of all these studies with the aglycone has been questioned.[6] Thus it was proposed that the mechanistic studies performed *in vitro* should be repeated with the main conjugated forms found in plasma. These studies have been limited by the lack of commercially available compounds for several years. The information available about the *in vitro* effects of these metabolites indicates that they are less active than the parent compounds and often totally inactive. For instance, in contrast to the aglycones,[7] glucuronidated and sulfated metabolites lack a direct acute vasodilator effect in isolated arteries and they have only a partial effect in preventing acute endothelial dysfunction.[8] Therefore, the suggestion that the conjugated metabolites are the active forms responsible of the biological effects *in vivo* is not fully supported by the present evidence. Thus the flavonoid paradox might be restated as follows: *Oral quercetin exerts biologically demonstrable systemic effects, but its circulating forms in plasma after oral administration show weak activity in vitro*.

Despite quercetin and isorhamnetin aglycones not being found in plasma, they are present in considerable amounts in tissues, together with variable amounts of conjugated metabolites.[9] In addition, glucuronoconjugated metabolites of quercetin can be hydrolyzed by β-glucuronidase, releasing the parent aglycone.[10] Hydrolysis of flavonoid glucuronides was found by and Shimoi *et al*. in human neutrophils[11] and by Lee-Hilz *et al*. in carcinoma cell lines,[12] who suggested that the activity of flavonoid metabolites depends on their deconjugation *in situ*. Terao *et al*., based on these and other indirect evidences,[13] proposed that glucuronide conjugates of quercetin function not only as detoxified metabolites but also as precursors of the bioactive hydrophobic aglycone.[14]

In order to understand the flavonoid paradox we raised some fundamental questions:[15]

1. Are quercetin glucuronides active *in vivo*? To address this issue, quercetin-3-glucuronide was injected intravenously (1 mg kg^{-1}) in rats (i.e. directly into the blood, where it is found after oral administration of quercetin), and blood pressure was monitored continuously. It must be borne in mind that this metabolite was totally inactive as an acute vasodilator *in vitro* in either large arteries[8] or small mesenteric arteries (unpublished data). Interestingly, *in vivo*, quercetin-3-glucuronide led to a progressive decrease in blood pressure over time. Thus the glucuronide in plasma was able to mimic the effects of oral quercetin, suggesting that the glucuronide might be responsible for the effects of the aglycones. However, we must note that the effect was not immediate; it was only significant 2 h after its administration, and it was more persistent (nearly 8 h) than the presence of the compound itself in plasma.
2. Are quercetin glucuronides deconjugated *in vitro* at the target tissues? We used the isolated perfused mesenteric bed as a vascular model and the glucuronide was continuously recirculated with the aid of a peristaltic pump.[15] The aglycone slowly appeared in the recirculated solution (detectable after 1 or 2 h and increased over 6 h), in parallel with a decrease of the glucuronide concentration. The aglycones could be also found in the tissue at 3 and 6 h. These data indicate that quercetin-3-glucuronide can be deconjugated at the vascular wall. Moreover, the enzyme β-glucuronidase was expressed in the vascular wall and its activity was measured.
3. Do the conjugates exert effects *in vitro* when incubated for sufficient time to be deconjugated? When an isolated mesenteric arterial ring was incubated with quercetin-3-glucuronide for 3 h the vasoconstriction induced by phenylephrine was inhibited, whereas incubation for 30 min was without effect.[15]
4. Does inhibition of deconjugation prevent the effects of the glucuronides? We used the classical inhibitor of β-glucuronidase saccharolactone which had been previously found to inhibit quercetin–glucuronide deconjugation in liver extracts.[10] This drug prevented both deconjugation in the perfused mesenteric bed and the long-term effects of glucuronides in the isolated mesenteric arterial ring.[15] All these results support that the circulating glucuronides in plasma behave as quercetin carriers and that the aglycone released in the target organs after deconjugation is the final effector (Fig. 1).

We further studied the flavonoid paradox in an animal model of disease: the spontaneously hypertensive rat (SHR).[16] We found that the blood pressure-lowering effects of intravenous quercetin-3-glucuronide were dose dependent, this metabolite being effective at doses as low as 0.2 mg kg^{-1}. Isorhamnetin-3-glucuronide was also effective *in vivo*, while the third major plasma metabolite, quercetin-3′-sulfate, was without effect. These *in vivo* effects of quercetin-3-glucuronide could be prevented by inhibiting deconjugation with saccharolactone. Finally, we analyzed whether the deconjugation process had a physiological role, i.e. if it was involved in the effects of quercetin when administered orally. In fact, inhibition of β-glucuronidase with saccharolactone fully prevented the antihypertensive effects of oral quercetin.[16] These results indicate that conjugation and subsequent deconjugation are required for the effects of orally administered quercetin.

Taken together, the current available evidence indicates that oral quercetin is partially absorbed in the intestine; it is extensively metabolized into glucurono- and sulfo-conjugated metabolites, which are the circulating forms in plasma, and glucurono-, but not sulfo-conjugates, can be hydrolyzed at the vascular level, yielding the aglycone, which can be accumulated in tissues (Fig. 1). Thus conjugation is a reversible process and, at least regarding the vasodilator and antihypertensive effects, the conjugation–deconjugation cycle appears to be an absolute requirement. Glucuronidated derivatives transport quercetin and deliver the free aglycone *in situ* by deconjugation. Deconjugation appears to be a slower process than conjugation. Because quercetin is more lipophilic it may remain trapped in the tissues, interacting with phospholipid bilayers of cellular and subcellular

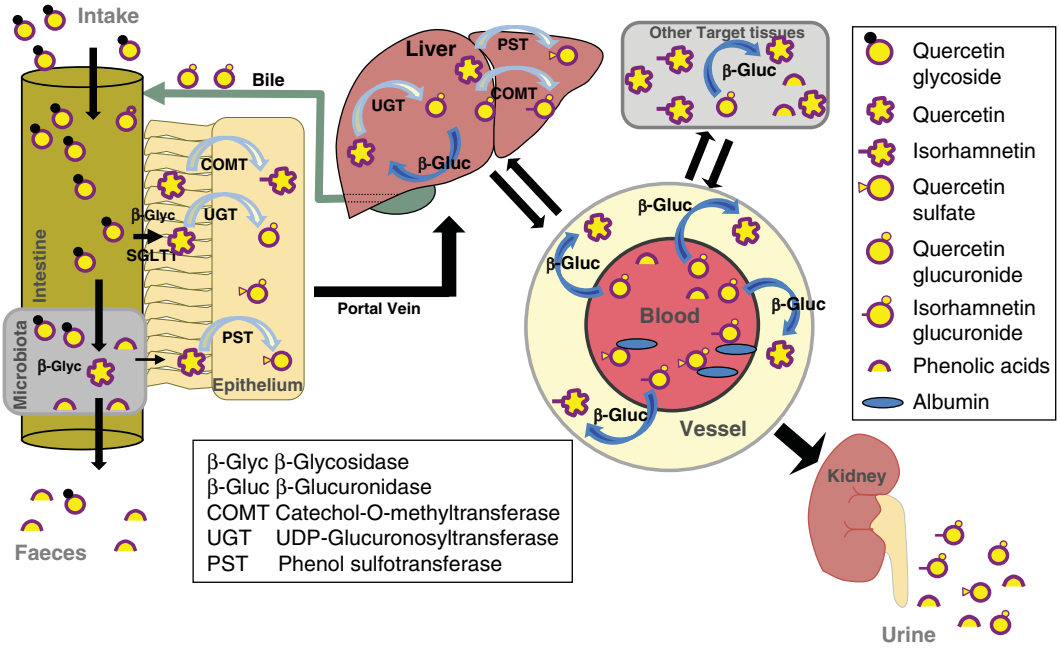

Figure 1. Schematic representation of the pharmacokinetics of quercetin.

membranes, and any quercetin released from the tissues may be re-conjugated again in the liver, explaining the low plasma levels. Yet some effects might be attributed to the conjugated metabolites themselves and it remains to be demonstrated whether deconjugation is also necessary for other important actions of flavonoids, especially the antiatherogenic effects. In addition, a role for other metabolites, especially those produced by the intestinal microbiota which may also enter the systemic circulation, should be assessed.[17]

Some practical considerations arise from the above. The most important one is that, because quercetin appears to be the final effector, several hundreds of mechanistic studies that have been performed with quercetin aglycone *in vitro* are not useless. Second, in studies analyzing the effects of conjugated metabolites *in vitro*, the presence and activity of β-glucuronidase of the cell type studied may be essential and the time factor must be also taken into account, because deconjugation is a slow process. Third, because the vessel wall is able to deconjugate glucuronides and these metabolites must cross this barrier to reach any tissue, it is expected that the aglycone can get into the tissues independently of the β-glucuronidase activity of non-vascular cells in the organ. In fact, in all tissues analyzed the aglycone was found but specific β-glucuronidase activity was not correlated with the proportions of deconjugated flavonols in the various tissues.[9] Fourth, saccharolactone is a useful tool to analyze the role of the conjugation–deconjugation process both *in vivo* and *in vitro*. Fifth, β-glucuronidase activity may influence the effectiveness of quercetin and this may be genetically determined. For example, β-glucuronidase activity was higher in transgenic mice expressing apoE3 compared to those expressing apoE4,[18] and this might be related to the decreased systolic blood pressure induced by quercetin in patients with an apoE3 genotype, whereas no significant effect was observed in the apoE4 group. Moreover, the β-glucuronidase activity is increased by bacterial endotoxin,[11] suggesting that quercetin may be more active under inflammatory conditions, such as in injured human aorta with atherosclerotic plaques.[13] Finally, similar catalytic efficacies were obtained for deconjugation of quercetin *O*-glucuronides substituted at different positions[19] and for deconjugation of other flavonols or isoflavonoids,[10] suggesting that all the above may also be valid for flavonoids different from quercetin.

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