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EFFECT OF VARIOUS FLAVONOIDS ON LYSOSOMES SUBJECTED TO AN OXIDATIVE OR AN OSMOTIC STRESS

THIERRY DECHARNEUX, FRANZ DUBOIS, CHRISTOPHE BEAULOYE, SIMONE WATTIAUX-DE CONINCK and ROBERT WATTIAUX*

Laboratoire de Chimie Physiologique, Facultés Universitaires Notre-Dame de la Paix, 61, rue de Bruxelles, B.5000 Namur, Belgium

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Abstract—When a light mitochondrial fraction (L fraction) of rat liver is incubated in the presence of an oxygen free radical generating system (xanthine–xanthine oxidase), the free activity of *N*-acetylglucosaminidase (NAGase) increases as a result of the deterioration of the lysosomal membrane. Various flavonoids are able to prevent this phenomenon, others are ineffective. Comparative activity studies suggest the importance of the presence of two OH groups in orthosubstitution in the B ring and of an OH in the 3 position. Flavan-type flavonoids behave like their related flavonoids; *d*-catechin also opposes lysosome disruption. Kaempferol, quercetin, 7,8-dihydroxyflavone and *d*-catechin inhibit lipoperoxidation occurring in an L fraction incubated with the xanthine oxidase system as ascertained by malondialdehyde (MDA) production. For kaempferol and quercetin, such an inhibition parallels the prevention of NAGase release; this is not the case for the two other compounds where inhibition of NAGase release takes place at a flavonoid concentration lower than that required to oppose MDA production. Morphological observations performed on purified lysosomes confirm the biochemical results. Some flavonoids are also able to prevent release of NAGase caused by the incubation of an L fraction in isoosmotic glucose. Only flavone and hydroxyflavones are effective. It is proposed that the protective effect of flavonoids on lysosomes subjected to oxygen free radicals does not only originate from their scavenger and antilipoperoxidant properties; a more direct action on lysosomal membrane making it more resistant to oxidative aggression has to be considered. The prevention by some flavonoids of lysosome osmotic disruption in isoosmotic glucose could be the result of an inhibition of glucose translocation through the lysosomal membrane.

The lysosome membrane prevents hydrolases associated with lysosomes from having access to molecules present in the surrounding medium. Its deterioration could be responsible for the degradation processes that take place in cells in various pathological situations [1]. The search for substances that could protect the membrane of lysosomes against agents causing its alteration is interesting from a physiopathological and a pharmacological point of view.

Recently, we have found that an extract of *Ginkgo biloba* used in venous pathology protected lysosomes exposed *in vitro* to oxygen free radicals and to osmotic stress. As such an extract is particularly rich in flavonoids, we decided to investigate the effect of these molecules on lysosomes subjected to the same aggressions. Results presented here indicate that certain flavonoids protect the lysosomal membrane against oxidative and osmotic damages, others are inefficacious. By comparing the different molecules we used, it appears that some structural requirements need to be fulfilled by a flavonoid to affect the response of lysosomes to oxygen free radicals and to lack of osmotic balance.

MATERIALS AND METHODS

Experiments were performed on male Wistar rats weighing 200–250 g. Liver light mitochondrial fractions (L fractions†) were prepared according to de Duve *et al.* [2].

The sensitivity of lysosome membrane to oxygen free radicals was assessed by incubating L fractions at 37° in a medium consisting of 0.01 M Tris–HCl buffer pH 8, 0.25 M sucrose, 0.5 mM xanthine, 2 mM ADP, 0.2 mM $FeCl_3$, 18 mU xanthine oxidase (Sigma Chemical Co., St Louis, MO, U.S.A.) in a volume of 1 mL and 10 μL of dimethyl sulfoxide (DMSO) or of the flavonoid solution in DMSO. After incubation aliquots were samples to measure free and total activities of *N*-acetylglucosaminidase (NAGase) as described by Jadot *et al.* [3]. In some experiments a non-enzymatic procedure to generate free radicals was used: L fractions were incubated at 37° in the presence of 0.01 M Tris–HCl pH 8, 0.25 M sucrose, 10 μM $FeCl_2$ and 0.2 mM ascorbic acid in a volume of 1 mL.

The formation of malondialdehyde (MDA) was followed by mixing 0.5 mL of the sample to be analysed with 20 μL of 2% terbutyl hydroxytoluene and 1 mL of a 0.3% thiobarbituric acid solution in 15% trichloracetic acid. The mixture was kept at 80° for 15 min; after centrifugation, the amount of MDA–thiobarbituric acid complex was determined by measuring the absorbance of the solution at 531 nm.

Osmotic stress was induced by incubating the organelles at 25° in a 1 mL mixture of 1 mM Tris–HCl, pH 7.4, 0.25 M glucose and 10 μL of DMSO or of the flavonoid solution in DMSO. Incubation

* Corresponding author. Tel. (32) 81 72 4268; FAX (32) 81 72 42 72.

† Abbreviations: L fraction, light mitochondrial fraction; MDA, malondialdehyde; NAGase, *N*-acetylglucosaminidase; DMSO, dimethyl sulfoxide.

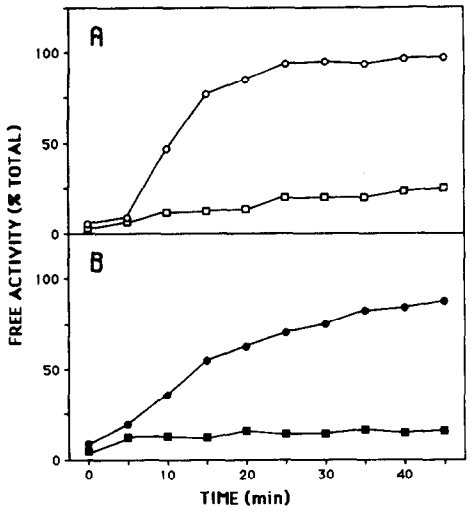

Fig. 1. Effect of the xanthine–xanthine oxidase reaction (A) and of isoosmotic glucose (B) on the free activity of NAGase. L fractions were incubated for increasing lengths of time at 37° in a medium consisting of 0.01 M Tris–HCl buffer pH 8, 0.25 M sucrose, with (○) or without (□) 0.5 mM xanthine, 2 mM ADP, 0.2 mM $FeCl_3$, 18 mU xanthine oxidase (A); or at 25° in 0.25 M glucose (●) or 0.25 M sucrose (■), 1 mM Tris–HCl pH 7.4 (B). After incubation aliquots were sampled to measure free and total activities of *N*-acetylglucosaminidase. Free activity is given as percentage of total activity. Total activity was 2.35 (A) and 2.15 (B) U/g fresh weight.

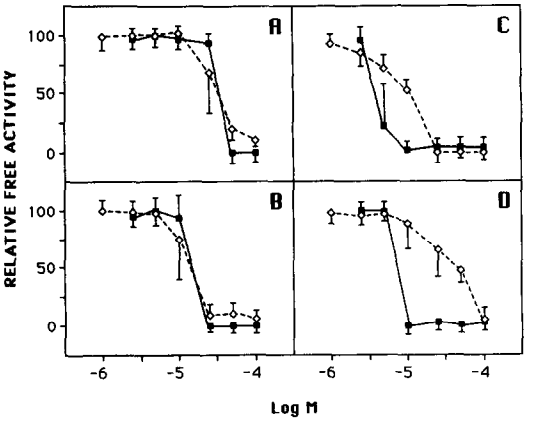

Fig. 3. Free NAGase and MDA production in the presence of kaempferol (A), quercetin (B), 7,8-dihydroxyflavone (C) and *d*-catechin (D). L fractions were incubated at 37° for 24 min in the presence of 0.01 M Tris–HCl buffer pH 8, 0.25 M sucrose, 0.01 mM $FeCl_2$, 0.2 mM ascorbic acid and the flavonoid at the indicated concentration. Aliquots were then sampled for determination of NAGase free and total activities and for measurement of MDA production as described in Materials and Methods. For result presentation, see legend to Fig. 2. (■) NAGase free activity; (◇) MDA. Total activity was 2.32 ± 0.15 (A), 2.40 ± 0.10 (B), 2.28 ± 0.12 (C) and 2.38 ± 0.11 (D) U/g fresh weight.

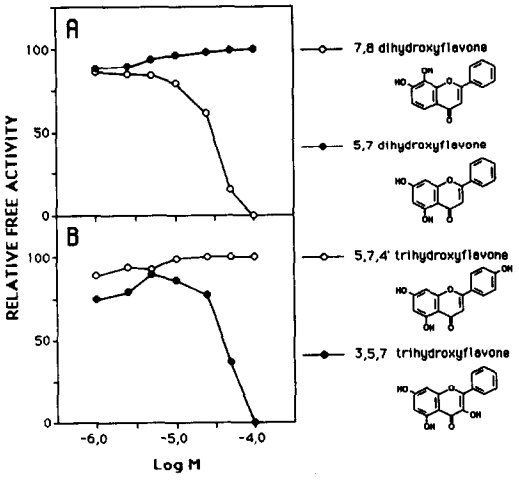

Fig. 2. Effect of dihydroxy (A) and trihydroxy (B)-flavones on lysosomes exposed to a free radical generating system. L fractions were incubated under the conditions described in the legend to Fig. 1. for 24 min at 37°, in the presence of various concentrations of flavonoids. Ordinate: relative free activity of NAGase, i.e. percentage of the free activity measured in the absence of flavonoids. Abscissa: flavonoid concentrations (log). Total activity was 2.20 (A) and 2.41 (B) U/g fresh weight.

at 25° was chosen because lysosome activation by an osmotic stress is too rapid at 37°. After incubation, samples were removed to determine free and total activities of NAGase [3].

For morphological examinations, lysosomes were purified in a Nycodenz gradient according to Wattiaux and Wattiaux-De Coninck [4] and processed for electron microscopy by the method of Baudhuin *et al.* [5] as described by Wattiaux *et al.* [6].

RESULTS

Incubation of lysosomes with the xanthine–xanthine oxidase system

As shown in Fig. 1A, when an L fraction is incubated for increasing lengths of time in the presence of the xanthine oxidase system, the free activity of NAGase rapidly increases, indicating an alteration of the lysosomal membrane. The effect of flavonoids on this process was investigated by determining the NAGase free activity of L fractions kept for 24 min in the presence of the oxygen free radical generating system without or with flavonoids at concentrations ranging from 10^{-6} to 10^{-4} M. The results are summarized in Table 1. They show that some flavonoids protect lysosomes against the oxidative agression. A structural requirement is the presence in these molecules of at least two OH groups: flavone and monohydroxyflavone are inefficient. The importance of two OH groups on orthosubstitution is illustrated in Fig. 2A where a comparison between 7,8- and 5,7-dihydroxyflavone is exhibited: the first compound is a good protector,

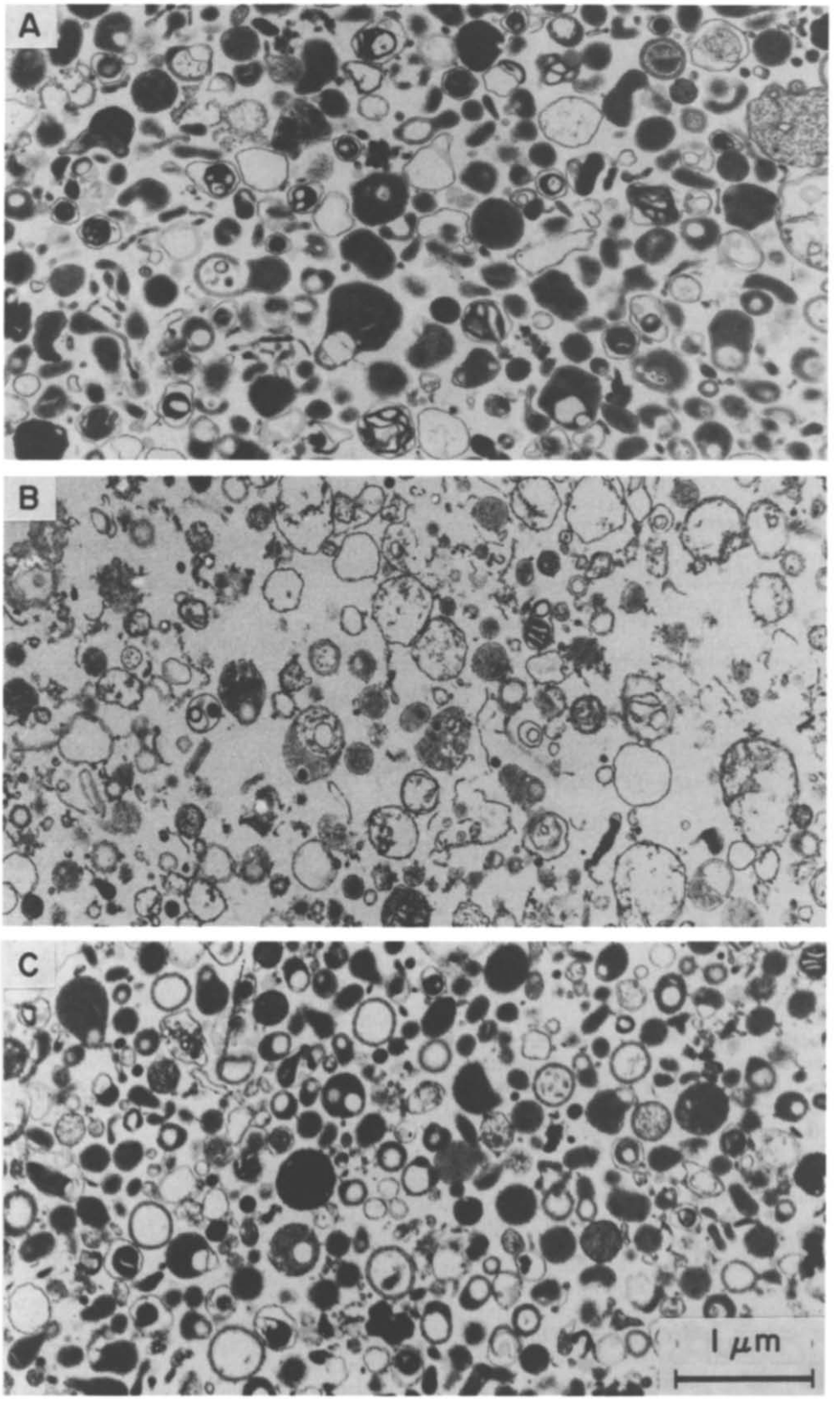

Fig. 4. Morphological aspect of a purified lysosome preparation. Lysosomes purified in a Nycodenz gradient were examined in the electron microscope before (A) or after incubation in the presence of the xanthine–xanthine oxidase system as described in the legend to Fig. 1, in the absence (B) or presence (C) of 0.05 mM *d*-catechin.

Table 1. Effect of flavonoids and *d*-catechin on lysosomes subjected to oxygen free radicals

I	A	B
7,8-Dihydroxyflavone	+	−
3,5,7-Trihydroxyflavone (galangin)	−	+
3,7,3′,4′-Tetrahydroxyflavone (fisetin)	+	+
3,5,7,4′-Tetrahydroxyflavone (kaempferol)	−	+
3,5,7,3′,4′-Pentahydroxyflavone (quercetin)	+	+
3,5,7,2′,4′-Pentahydroxyflavone (morin)	−	+
3,5,7-Trihydroxy-4′-methoxyflavone (kaempferide)	−	+
3-Rutinoside of quercetin (rutin)	+	−
3,5,7,3′,4′-Pentahydroxyflavanone (taxifolin)	+	+
3,5,7,3′,4′-Flavanpentol (*d*-catechin)	+	+
II	**A**	**B**
Flavone	−	−
6-Monohydroxyflavone	−	−
7-Monohydroxyflavone	−	−
5,7-Dihydroxyflavone (chrysin)	−	−
5,7,4′-Trihydroxyflavone (apigenin)	−	−
5,7,3′-Trihydroxy-4′-methoxyflavone (diosmetin)	−	−
5-Hydroxy-7,3′,4′-ethoxy-3-rutinoside (troxerutin)	−	+
Flavanone	−	−
5,7,4′-Trihydroxyflavanone (naringenin)	−	−
5,7,3′-Trihydroxy-4′-methoxy-7-rutinoside flavanone (hesperidin)	−	−

L fractions were incubated for 24 min at 37° in a medium consisting of 0.01 M Tris–HCl buffer pH 8, 0.25 M sucrose, 0.5 mM xanthine, 2 mM ADP, 0.2 mM $FeCl_3$, 18 mU xanthine oxidase (Sigma) in the presence of various concentrations of flavonoids ranging from 10^{-6} to 10^{-4} M. After incubation, aliquots were sampled to measure free and total activities of NAGase.

I: Substances that at 10^{-4} M concentration cause 80–100% inhibition of the NAGase free activity increase; II: substances that at 10^{-4} M concentration do not cause an inhibition greater than 20%.

A: Presence (+) or absence (−) of two OH groups in orthoposition; B: presence (+) or absence (−) of a 3OH group.

the second one is without effect. The presence of an OH group in the 3 position of the pyrane ring is also favorable as exemplified in Fig. 2B: addition of a third OH to 5,7-dihydroxyflavone confers an activity on the molecule if it takes place in the 3 position (galangin) but not in the 4′ position (apigenin). The importance of these two structural characteristics, orthosubstitution and OH in the 3 position, is emphasized by the fact that all the active molecules have at least one of these characteristics (Table 1). In addition, results suggest that flavonoids and flavan-type flavonoids behave similarly: flavanone, naringenin and hesperidin are inactive like the corresponding flavonoids: flavone, apigenin and diosmetin. On the other hand, taxifolin is as active as quercetin.

As some flavonoids are inhibitors of xanthine oxidase [7], we tried to determine whether the protection of the lysosomal membrane that we observed was not related to that inhibitory effect. We found that there was no relationship between the capacity for a flavonoid to prevent lysosome deterioration and to inhibit acid uric production by xanthine oxidase. Moreover, the protective effect of flavonoids on lysosomes takes place also when oxygen free radicals are produced by a non-enzymatic system consisting of ascorbic acid and ferrous chloride (not shown).

A well-known effect of oxygen free radicals is the lipid peroxidation they can induce in biological membranes. Flavonoids could protect the lysosomal membrane by inhibiting peroxidation. This hypothesis was tested by measuring the MDA production, the indicator of lipid peroxidation, in L fractions subjected to oxygen free radicals and by determining the effect of flavonoids on this phenomenon together with their effect on NAGase free activity. Figure 3 illustrates results obtained with kaempferol, quercetin, 7,8-dihydroxyflavone and *d*-catechin. The four substances protect lysosomes and inhibit MDA production. A correlation between the two phenomena is manifest for kaempferol and quercetin; on the other hand, significant differences are observed in the case of 7,8-dihydroxyflavone and of *d*-catechin with respect to their effect on MDA production and on NAGase free activity. For the last compound, total inhibition of MDA production requires a concentration 10 times higher than that needed to prevent totally NAGase latency loss. It is to be noted that 7,8-dihydroxyflavone is apparently more efficient than *d*-catechin in preventing MDA production. At present, we have no explanation for such a difference.

Table 2. Effect of flavanoids and *d*-catechin on lysosomes subjected to osmotic stress

I
Flavone
6-Monohydroxyflavone
7-Monohydroxyflavone
7,8-Dihydroxyflavone
5,7,4′-Trihydroxyflavone (apigenin)
3,7,3′,4′-Tetrahydroxyflavone (fisetin)
3,5,7,4′-Tetrahydroxyflavone (kaempferol)
3,5,7,3′,4′-Pentahydroxyflavone (quercetin)
3,5,7,2′,4′-Pentahydroxyflavone (morin)
II
5,7-Dihydroxyflavone (chrysin)
3,5,7-Trihydroxyflavone (galangin)
3,5,7-Trihydroxy-4′-methoxyflavone (kaempferide)
5,7,3′-Trihydroxy-4′-methoxyflavone (diosmetin)
3-Rutinoside of quercetin (rutin)
5-Hydroxy-7,3′,4′-ethoxy-3-rutinoside (troxerutin)
Flavanone
5,7,4′-Trihydroxyflavone (naringenin)
3,5,7,3′,4′-Pentahydroxyflavone (taxifolin)
5,7,3′-Trihydroxy-4′-methoxyflavanone-7-rutinoside (hesperidin)
3,5,7,3′,4′-Flavanpentol (*d*-catechin)

L fractions were incubated at 25° for 30 min in 0.25 M glucose and 1 mM Tris–HCl buffer pH 7.4. After that free and total activities of NAGase were measured.

I: Substances that at 10^{-4} M concentration cause 80–100% inhibition of the NAGase free activity increase; II: substances that at 10^{-4} M concentration do not cause an inhibition greater than 20%.

Morphological observations confirm the biochemical results. Lysosomes were purified in a Nycodenz gradient [4] and subjected to the xanthine oxidase system in the absence or presence of 50 μM catechin, and the organelles were then processed for electron microscopy examination according to Baudhuin *et al.* [5]. In the absence of treatment (Fig. 4A), lysosomes appear quite normal as described previously [6]. After incubation with xanthine oxidase (Fig. 4B), a gross deterioration of numerous lysosomes is observed, characterized by the presence in the preparation of empty structures and membrane fragments. It is obvious that these alterations are largely prevented when incubation with the free radical generating system is performed in the presence of catechin (Fig. 4C).

Osmotic disruption of lysosomes by glucose

Incubation of a lysosome preparation in isoosmotic glucose causes a release of NAGase (Fig. 1B). Glucose enters the lysosomes and induces an osmotic unbalance between the organelle matrix and the external medium. As a result, water penetrates the granules, and causes their swelling and the rupture of their membrane [8]. The effect of flavonoids (10^{-6}–10^{-4} M) was tested by measuring the NAGase free activity of an L fraction maintained for 30 min in the glucose medium. Results are summarized in Table 2. Only flavone and hydroxyflavones are able to prevent osmotic disruption of lysosomes. Introduction of a methoxygroup (kaempferol → kaempferide) or of a glycosyl (quercetin → rutin) and saturation of the C ring (apigenin → naringenin) suppress the lysosome protection afforded by the flavonoid. Catechin is also inefficacious.

DISCUSSION

Our results show that some flavonoids are able to prevent the deterioration of lysosomes exposed to an oxygen free radical generating system and to osmotic stress.

Flavonoids are free radical scavengers. It may be asked if the protective action of these molecules on lysosomes originates from their scavenger capacity. Recently, Sichel *et al.* [9] have tried to establish a relationship between the structure of several flavonoids and their scavenger capacity. A limited correlation exists between the scavenging efficiency found by these authors and the capacity to prevent lysosome deterioration that we have described. It concerns the requirement of a minimum number of OH groups, flavone and flavanone are poor scavengers, and the importance of OH in the 3 position. On the other hand, even though the presence of a hydroxyl group in the B ring seems essential for scavenging capacity [9], it is not necessary for lysosomal membrane protection: 7,8-dihydroxyflavone and galangin which are devoid of substitution in the B ring protect lysosomes; apigenin which possesses a 4′OH is inefficacious. It is also to be noted that according to Pincemail *et al.* [10], hesperidin inhibits $OH^{\cdot}$ production in the xanthine–xanthine oxidase system but was unable to protect lysosomes in our experiments. We have also shown that there is apparently no correlation between the inhibition of lipoperoxidation by some flavonoids and their protective effect on lysosomes. Therefore, it is improbable that only the scavenger properties of flavonoids and their antilipoperoxidant activity are responsible for the protection these molecules afford to lysosomes subjected to oxygen free radicals. A more direct action on the membrane making it more resistant to oxidative agression has to be considered. According to Weglicki *et al.* [11], enhanced lysosome phospholipid degradation occurs when these organelles are incubated in the presence of a free radical generating system owing to an increased sensitivity of peroxidized lipids to endogenous lipases. A plausible hypothesis would be that flavonoids protect lysosomes by making lysosome membrane lipids more resistant to lipases or by inhibiting these enzymes.

The mechanism by which flavonoids prevent the disruption of lysosomes by glucose is not known, but they could inhibit the penetration of glucose into the organelles or make the membrane more resistant to swelling. It is worthwhile mentioning that the concentration range where an effect of flavonoids is observed (10^{-5}–10^{-4} M) is analogous to that required by phloretin, a specific inhibitor of glucose transport, to prevent lysosome disruption caused by isoosmotic glucose [3]. This suggests that flavonoids could be inhibitors of glucose translocation.

The effects of flavonoids on lysosomes have to be added to the numerous biological effects previously described for these molecules: inhibition of macromolecule synthesis [12], of reverse transcriptase [13], of protein kinase [14] and of ATPase [15]. These properties could be responsible for the pharmacological effects of certain plant extracts rich in these compounds. With respect to lysosome protection, the presence in these extracts of 7,8-dihydroxyflavone, fisetin, kaempferol, quercetin and morin could be particularly interesting since these flavonoids increase the resistance of lysosomes to oxidative as well as to osmotic stress.

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