Molecular mechanisms involved in the cardiovascular and neuroprotective effects of anthocyanins

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ABSTRACT

Anthocyanins are the main group of natural hydrosoluble pigments in plants. They introduce colouring to foods, with colours ranging from blue to red and orange. Nowadays, their importance for the Food and Pharmaceutical industries is mainly based in the existing scientific work evidencing their beneficial effects on the prevention of cardiovascular diseases and neurological conditions. Different mechanisms have been shown to be involved in those effects. The most consistent ones are related to their antihypertensive and endothelium protective activities, antiatherogenic activity and their interaction with the estrogenic receptor. In some of the existing work, studies on structure–activity relationship have been done, showing that modifications on the structure of anthocyanins, besides having an effect on their colours, have a clear incidence on their interaction with different steps in the principal pathways related to these diseases. Therefore, different colours might show different molecular mechanisms. However, in a normal diet most of these compounds are present simultaneously and, thus; they can act by different mechanisms but can rise to a common final action. Design of new food product or food supplements should take these potential synergies into consideration.

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Introduction

Anthocyanins are natural pigments present in many fruits and some vegetables. These polyphenolic substances are glycosides of polyhydroxy and polymethoxy derivatives of 2-phenylbenzopyrylium or flavilium salts. The most common anthocyanidins in plant foods are pelargonidin, cyanidin, peonidin, delphinidin, petunidin, and malvidin. Glycosylation and acylation of the aglycone moieties by different sugars and acids, at different positions, account for the broad structural diversity of these pigments (Fig. 1).

This red–orange to purple pigments have in common their interest to the food industry due to their use as natural colorants. This has led to many studies, in the past, having been aimed to clarify their stability and range of colours at different pH and temperatures. This way, it has been shown that minor structural differences between anthocyanins produce big differences in the colour of the final food product. In fact it has been largely assumed that acylation of anthocyanins induce the stabilization of the structure of anthocyanin molecules that increase their resistance to changes in pH and temperature. However recently it has been shown that this is not always true and tha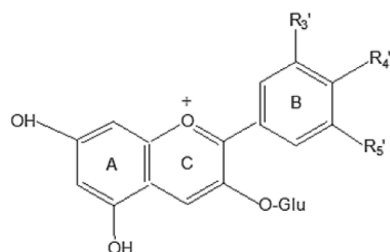t not in every case acylation of the parent anthocyanin means an increase on its stability [24]. Additionally the colours shown by anthocyanins depend largely on the pH at which they are dissolved. This way in strongly acidic media they tend to be orange or red while when in a less acidic media they are red–violet and they are blue only in strongly basic media [8].

But here our interest in anthocyanins is health related. Anthocyanins have proved in epidemiological and clinical studies being effective in preventing different diseases. For instance, it has been reported that a higher intake of anthocyanins is associated with a lower arterial stiffness [14]. Flow-mediated dilation (FMD)[1] has been shown to be increased after blueberries consumption in healthy men [19]. In epidemiological studies it has been shown that, in young women, high anthocyanin consumptions are associated with a decrease on the risk of myocardial infarction [2]. As for Parkinson disease, it has been shown that high anthocyanin consumption is associated with a reduction of the risk of developing this disease [7]. To better understand these potential effects it is

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[1] *Abbreviations used:* FMD, flow-mediated dilation; SAR, structure–activity relationship; TNF, tumour necrosis factor; TNFR2, TNF receptor-associated factor 2; NADPH, nicotinamide adenine dinucleotide phosphate; ACE, angiotensin-converting enzyme; ROS, reactive oxygen species; LBDs, ligand binding domains; PPARγ, peroxisome proliferator-activated receptor gamma.

ANTHOCYANINS	R'$_3$	R'$_4$	R'$_5$	Dietary sources*
Pelargonidin-3-glucoside	H	OH	H	Strawberries
Cyanidin-3-glucoside	OH	OH	H	Raspberries, blackberries, blackcurrants
Peonidin-3-glucoside	OCH$_3$	OH	H	Grapes
Delphinin-3-glucoside	OH	OH	OH	Blueberries, blackcurrants, beans
Petunidin-3-glucoside	OCH$_3$	OH	OH	Blueberries
Malvidin-3-glucoside	OCH$_3$	OH	OCH$_3$	Grapes, blueberries, beans

*Dietary sources (adapted from Neveu et al. [16])

Fig. 1. General structure and most common anthocyanins found in plant foods. (See above-mentioned references for further information.)

important to carry on mechanistical studies to know how this pigments act in the human body. Additionally, it has been recently proven that these anthocyanin breakdown metabolites are present in plasma in higher amounts and for longer periods that previously reported [4]. This is the reason for the inclusion of some data on the mechanisms involved in anthocyanin breakdown metabolites in this manuscript.

One could say that there is a similarity between the colour anthocyanins produce and the mechanisms by which they act; they are both depending on SAR (structure–activity relationship). And the same holds, of course, for their stability, their bioavailability and metabolism, their efficiency or their selectivity (Fig. 2). They are all structure dependent and thus can be modified by the inclusion of different sugars or other substituents in the structure of the molecule.

Fig. 1 shows the general structure of anthocyanins. They tend to have a minimum of 4 hydroxyl groups attached to the C6-C3-C6 structure making them flavonoic polyphenols. In general they are glycosylated in 3 position with different sugars, the most common being, glucose as monoside, followed by galactose, xylose and arabinose; but that can also be a di or tri glycoside. Additionally they can have glycosidic rests in positions 5 and 7 and can also be acetylated at different positions. This makes it possible to find in nature more than 500 anthocyanins. However in foods the most common ones are derived from the anthocyanidins pelargonidin, with just one hydroxyl group at 4′, cyanidin with two hydroxyl groups attached to the phenyl ring in positions 3′ and 4′ and delphinidin with 3 hydroxyl groups at 3′, 4′ and 5′. Then peonidin, petunidin and malvidin are mono or di methoxylated derivatives at different positions in the B ring of the molecule. Then again they
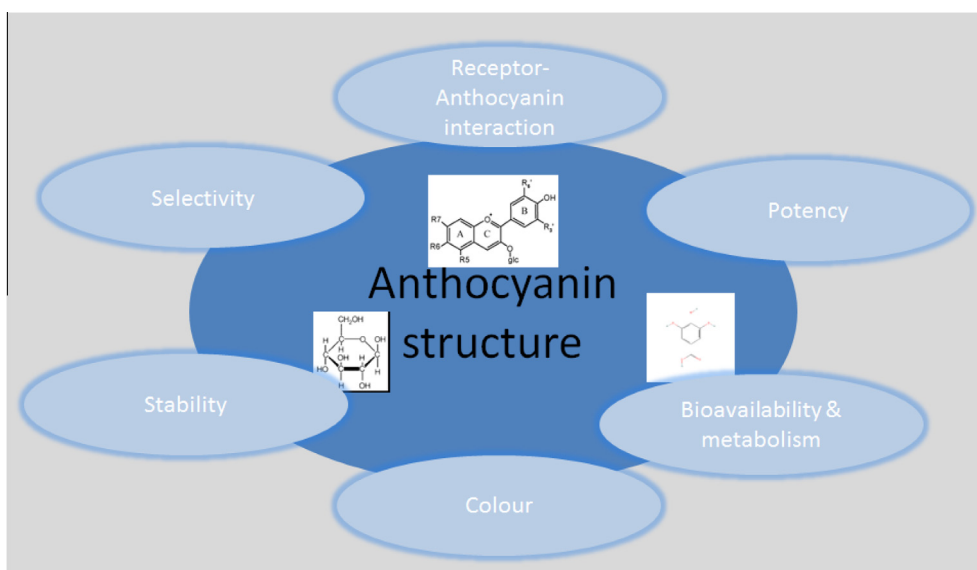

Fig. 2. Structure–activity relationship (SAR) affect anthocyanins stability, physicochemical characteristics and biological activity.

can be glycosylated at different positions to give the corresponding anthocyanins [3]. Well then, there are differences in colour between the different, most common, anthocyanins in plant foods, going from the orange–red pelargonidin-3-glucoside to the purple delphinidin. These structural differences have an impact in their colour but, as I will be showing from now, have also an impact on their interaction with molecular pathways that are involved in their potential health effects.

More concretely on those mechanisms related to conditions like cardiovascular disease, visual conditions or cognition related, which are the most promising ones in terms of the potential of this group of pigments to prevent them. These mechanisms are mainly related to the inflammatory response, the endothelial function, the fatty acid metabolism or glucose metabolism and their interaction with microflora.

Interaction with PPAR gamma-ABCA1 pathway: effects on lipid distribution

One of the first approaches to this matter was that of Xia and co-workers studying the effect of anthocyanins on the ABCA1 pathway. It has been shown that in older macrophages an abnormal polarization is caused by programmatic changes that lead to reduced expression of ATP binding cassette transporter ABCA1 [25]. Down regulation of ABCA1 reduce the ability of macrophages to effectively efflux intracellular cholesterol, which leads to higher levels of free cholesterol within senescent macrophages. This accumulation of cholesterol inside macrophages leads to angiogenesis which plays a central role in common age-associated disease such as atherosclerosis, cancer, and macular degeneration. Xia and co-workers showed some years ago that anthocyanins, more specifically cyanidin and peonidin 3 glucosides, are able to increase the efflux of cholesterol from macrophages in a dose response manner. They showed also in mouse peritoneal macrophages that this effect is mediated via the ABCAI pathway by up regulating its expression and that this happens through peroxisome proliferator-activated receptor gamma (PPARγ) activation. This effect was shown at a concentration of anthocyanin as low as 1 μmol/l; however no differences were shown between cyanidin and its methoxylated derivative peonidin.

More recently, it has been shown that effectively cyanidin interacts with PPARs subclasses. Being more effective the interaction with α and γ than that of δ/β [15]. Jia and co-workers also reported that this interaction takes place through the hydroxyl group at 4′ of cyanidin structure, explaining also the lack of differences encountered in the previous study between cyanidin and its methoxylated derivative peonidin, since both have their hydroxyl group at 4′ available.

The same authors showed that cyanidin significantly reduced cellular lipid concentrations in lipid-loaded steatotic hepatocytes. In addition, transcriptome profiling in lipid-loaded primary hepatocytes revealed that the net effects of stimulation with cyanidin on lipid metabolic pathways were similar to those elicited by hypolipidemic drugs such as lovastatin or fenofibrate. The authors conclude that cyanidin likely acts as a physiological PPARα agonist and potentially for PPARβ/δ and γ, and reduces hepatic lipid concentrations by rewiring the expression of genes involved in lipid metabolic pathways. This mechanism might well explain some of the results encountered in epidemiological and clinical studies showing the effect of an increased anthocyanin consumption on arterial stiffness [14] or cholesterol homeostasis [9].

CD40 is a member of tumour necrosis factor (TNF) receptor superfamily that provides activation signals to many cells. TNF receptor-associated factor 2 (TNFR2) directly binds to a membrane-distal CD40 cytoplasmic domain and is thought to play a pivotal role in the signalling pathways initiated by CD40. Xia and co-workers have found that anthocyanins Cy-3-g and Pn-3-g dose-dependently protect from CD40-induced proinflammatory signalling by preventing TRAF-2 translocation to lipid rafts through regulation of cholesterol distribution, which thereby may represent a mechanism that would explain, at least partially, the anti-inflammatory response of anthocyanins [25].

Interactions with NF-κB pathway: effects on inflammation

NF-κB plays a critical role in regulating the expression of a large number of genes involved in immune, inflammatory and apoptotic processes. NF-κB can be activated by different stimuli such as microbial products, proinflammatory cytokines, T and B cell mitogens and physical and chemical stresses. NF-κB in turn regulates the inducible expression of many cytokines, chemokines, adhesion molecules, acute phase proteins and anti-microbial peptides. In unstimulated cells, NF-κB is retained in the cytoplasm through its interaction with the inhibitory IkB proteins. Stimulation of cells with different inducers leads to the phosphorylation and subsequent degradation of the IkB proteins. Upon degradation of IkB, the free NF-κB enters the nucleus; however translocation of NF-κB to the nucleus by itself is not sufficient to regulate the transcription of target genes. Instead, specific phosphorylation of one of the NF-κB subunits, p65, is required for both efficient DNA-binding and transcriptional activity of the nuclear NF-κB.

An anthocyanin rich extract with up to 80% being cy-3-glu has been shown to affect NF-κB activity in LPS-stimulated BV2 microglia cells. In the study by Poulose and co-workers [18] cells were pre-treated with different concentrations of anthocyanins before LPS treatment. The authors showed that ikB and cytosolic p65 decreased after the treatment with LPS, and that this decrease was counteracted by the pre-treatment with this cyanidin rich extract. Similarly the nuclear p65 which increased after translocation due to LPS stimulation was decreased by the presence of cyanidin. This modification of Nf-κB activity was accompanied by a down-regulation of iNOS and COX-2, as showed by Poulose et al. [18].

In macrophages, it has been shown that from the five more abundant anthocyanidins, delphinidin, cyanidin, pelargonidin, peonidin and malvidin, only delphinidin and to a less extent cyanidin is able to down regulate COX2 expression. In this case, showing again that the effect is mediated via the NF-κB pathway with an induction of IkB-alpha and inhibition of translocation of NF-κB at 50 μM delphinidin [12].

The same has been shown in endothelial cells. In [26] work delphinidin and cyanidin were used to see the effect on oxLDL activation. The phosphorylation of p38MAPK was determined by the proportion of the phosphorylated and unphosphorylated p38MAPK expression. And the nuclear translocation of NF-κB (p65) was determined by the proportion of NF-κB (p65) expression in the nucleus and that in the cytoplasm. Both cyanidin and delphinidin were able to inhibit p38 phosphorylation and NF-κB translocation in a similar way.

When vascular smooth muscle cells are stimulated with angiotensin II, certain isoforms of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase get activated and augment the production of the very pro-oxidant species, superoxide. This effect is mediated by AT1-receptor in the cell membrane and will activate NF-κB pathway. This has been shown because different gens, like VCAM's, are known to be activated via NF-κB. This is one of the reasons why in our group we decided to measure the potential of different anthocyanins and their breakdown metabolites to inhibit one of the key components of the retin–angiotensin system, angiotensin-converting enzyme (ACE). We first assayed a series of

anthocyanins and their potential break-down metabolites at a fix concentration to see if there was any ACE inhibitory effect and then from those having a higher inhibitory potential we calculated the IC$_{50}$ [10,11]. This way we showed that all the anthocyanins assayed were more efficient than the phenolic and hydroxyphenolic acids. Delphinidin, cyanidin and pelargonidin were more active in inhibiting ACE and the two methoxylated anthocyanins malvidin and peonidin were less active. Even if the concentration needed to afford a 50% inhibition of ACE were quite high, especially if we compare them to the one of a known ACE inhibitor widely used in clinic such as Captopril. However we should have in mind, that the inhibition of ACE was significant at 10 μM for example in the case of delphinidin.

Interactions with Keap1-Nrf2 pathway: effects on cell survival

The transcription nuclear factor erythroid 2-related factor 2 (Nrf2) neutralize or detoxify both endogenous metabolites and environmental toxins. Nrf2 appears to function when released from its repressive redox-sensitive companion protein Keap1 by sensing cytoplasmic oxidative stress or some chemical agents. After translocation into the nucleus, Nrf2 stimulates transcription of genes encoding detoxifying and antioxidant enzymes, such as NADPH (nicotinamide adenine dinucleotide phosphate) quinone oxidoreductase1 (NQO1), GSH S-transferase (GST), hemeoxygenase-1 (HO-1), glutamate cysteine ligase (GLC) and peroxyredoxin I, GSH peroxidase, which contribute to cellular protection by removing reactive oxygen species (ROS) including superoxide anions, hydrogen peroxide and hydroxyl radical (Fig. 3). Speciale and co-workers have shown that cyanidin is able to induce Nrf2 in endothelial cells and that as a consequence there is an induction of hemeoxygenase-1 [22]. Concluding that this way cyanidin-3-glucoside might protect the cardiovascular system.

Inhibition of autophagy increase cell viability and decreased cell death. Zhang and co-workers have shown that pre-treatment of β cells with cyanidin attenuates oxidative stress-mediated autophagic cell death [27]. Anthocyanins activate the antioxidant transcription factor Nrf2, and Nrf2/HO-1 negatively regulate autophagy process, thus increasing viability of β cells. The authors showed this at a concentration as low as 1 μM. Furthermore, they demonstrated that autophagy also takes place in β cell grafts during the early post-transplantation phase. However, β Cells pre-treated with anthocyanins displayed decreased extent of autophagy after transplantation.

Estrogen receptor(ER): interaction with other pathways

NF-κB can inhibit the estrogenic receptor in different ways (Fig. 4). For example activation of Akt can inhibit the activity of forkhead box O3, FOXO3A, a protein that has an important role in the synthesis of estrogenic receptor. Besides the repression of the estrogenic receptor by NF-κB, ER is also able to repress NF-κB. This is done by two ways; one is by inhibiting the translocation of NF-κB and its accumulation in the cytosol of the cell which will lead to its partial degradation. This can be done via the prospahtidilinositol-3-kinase (PI3K) signalling pathway. A second mechanism by which the estrogenic receptor may inhibit NF-κB activity is by preventing the binding the transcriptional factor to the DNA. Estradiol for example has been shown to interact with different co-repressors and to compete for co-activators [21].

In our group we have assayed the ER binding affinity of anthocyanins and their metabolites in a radioactivity assay by measuring their ability to compete with 17-β-estradiol for the ER, alpha and beta, by scintillation counting [10,11]. These series of studies, as other involving anthocyanin molecular mechanisms of action, are, at least partially, sustained on previous results showing the ability of anthocyanins [6,28] and gallic acid [20,23] to be uptaken by cells.

In our experiments, pelargonidin-3-glucoside showed affinity for both receptors, with a relatively higher affinity for ERα (61.3 μM ± 0.7) than for ERβ (93.0 μM ± 0.8), whereas peonidin-3-glucoside only demonstrated affinity for ERα (64.4 μM ± 0.9) and delphinidin-3-glucoside only reasonable affinity for ERβ (63.2 μM ± 0.8). We also assayed the breakdown metabolites deriving from anthocyanins. Among the phenolic acid metabolites assayed, gallic acid showed affinity for ERβ (100.3 μM ± 0.9) but did not show affinity for ERα. It is interesting to note that delphinidin-3-glucoside and gallic acid, with similar structural features in the hydroxylation pattern of their B ring, showed affinity for ERβ, but not for ERα. Moreover, delphinidin-3-glucoside demonstrated a binding affinity for ERβ that is approximately 2-fold higher than that of gallic acid.

We also carried out docking studies for the anthocyanidins delphinidin, pelargonidin, peonidin, malvidin and cyanidin and their metabolites (gallic and protocatechuic acids); in the ligand binding domains (LBDs) of both ERα and ERβ. In general, docking predictions were in agreement with affinity data.

Taking genistein (binding mode) as a reference, our purpose was to study whether the docking protocol was able to predict both the binding poses for anthocyanidins and any binding preference that could suggest selectivity. Regarding the binding poses, the most favourable poses corresponded to docked orientations where hydroxyl bonds between hydroxyl groups and His524 (ERβ His476) and Arg394-Glu353 (ERβ Arg346-Glu305) are the main interactions, anchoring the ligand within the ligand binding domain of the estrogenic receptor. These interactions are crucial to give rise to a stable ligand-receptor complex that could account for the observed affinity. Regarding selectivity, no solutions were predicted for delphinidin by AutoDock when docked to ERα, while favourable binding orientations were found in ERβ. On the contrary, in the case of peonidin, only favourable poses were located in ERα. These results were in agreement with the experimental affinity values, which show ERβ selectivity for delphinidin and ERα selectivity for peonidin [10,11]. No binding poses were found for malvidin and cyanidin, in agreement with the absence of affinity, suggesting that these compounds are not suitable ER-ligands.

In the case of phenolic acids, which are smaller molecules, all showed ability to bind to different regions of the ligand binding domain. Our computational efforts were then directed towards the study of the putative binding of two molecules of gallic acid within the ligand binding domain for ER. AutoDock predicted that two units of both phenolic acids are able to interact simultaneously with the LBD, by adopting several orientations (Fig. 5). Thus, we found that this hypothesis was possible from a theoretical perspective. From the study of interactions and predicted binding energies, it can be concluded that gallic acid binds with higher affinity to both ERs, compared with protocatechuic acid. Regarding selectivity, similar binding energy values were predicted for both ERα/ERβ receptors. With these theoretical results, selectivity cannot be considered [10,11].

The proteasome system

Yet, another possible mechanism by which anthocyanins can regulate NF-κB pathway is the proteasome system. In fact, proteasome inhibitors are thought to prevent IkB degradation and thus will prevent NF-κB activation. Proteasome activity controls the degradation of cellular proteins and is closely involved in signal transduction, development, cell cycle progression, antigen processing,
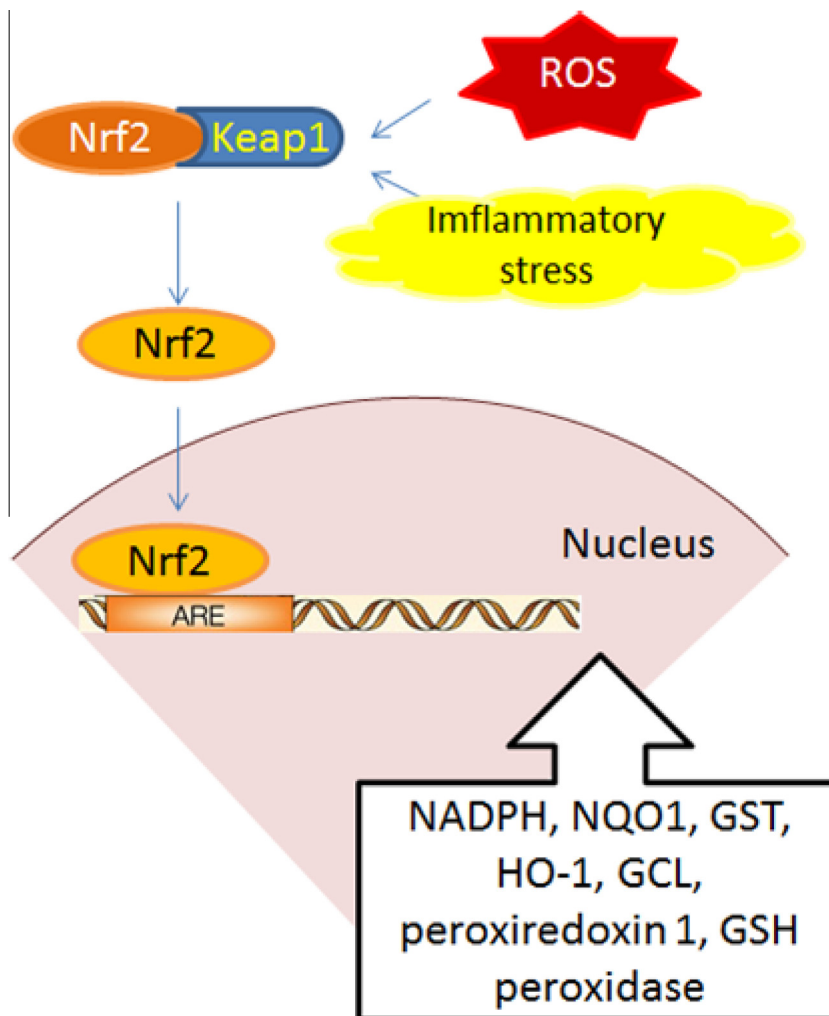
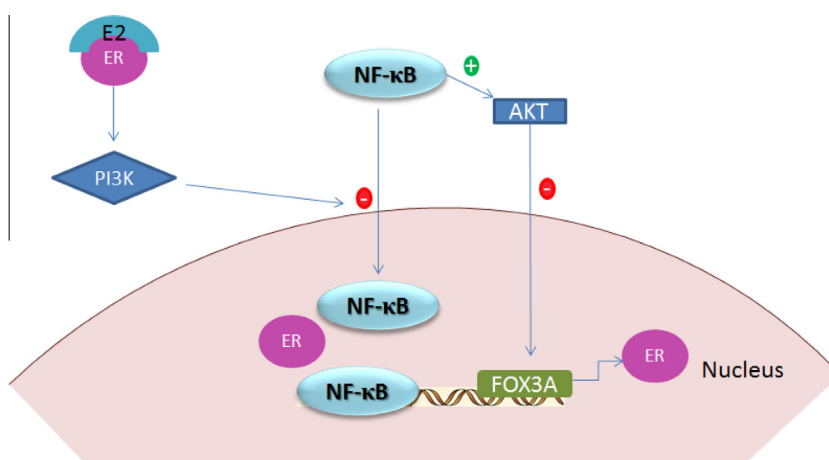**Fig. 3.** Scheme of Nrf2 pathway.

Fig. 4. Trans-repression of the oestrogen receptor and NF-κB.

immune response, and inflammation plus protection from oxidative stress relevant to different diseases.

The study by Dreiseitel and co-workers was conceived to examine anthocyanins' and anthocyanidins', in vitro impact on the chymotrypsin-like (ChT-L) proteasome activity. The work, carried out in a Human promyelocytic leukemia cells, HL-60 cells, has shown that anthocyanidins and anthocyanins are able to inhibit the chymotrypsin-like enzyme proteasomes activity [5]. The authors showed this way that the most active anthocyanidins in this model were pelargonidin and its methoxylated metabolite Kaempfiridinidin. The inclusion of another hydroxyl group in 3′ position, as in cyanidin showed a decrease in activity, while its methoxylation
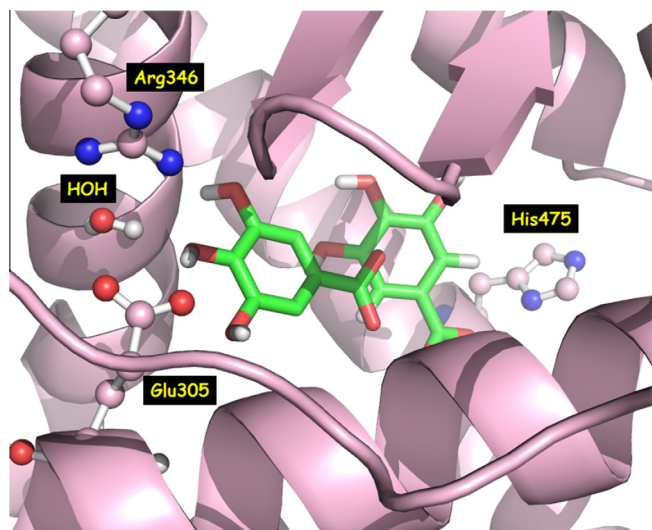
Fig. 5. Model of two molecules of gallic acid inside the ERβ ligand-binding domain.

increased it. And the same occurred when delphinidin was methoxylated to cause an increase in activity, as in the case of petunidin. When comparing aglycons with glycosides, in general monoglycosilation in 3 position of the anthocyanidin showed an increase in chymotrypsin-like proteasome activity while diglycosilation in 3 and 5 showed a decrease in activity.

Interaction with the (PI3K)/Akt pathway

Inactivation of the phosphatidylinositol 3 kinase (PI3K)/Akt pathway prevent apoptosis in an endothelial cell culture model. Paixao and co-workers have shown that pelargonidin, cyanidin and delphinidin are able to prevent apoptosis by inhibiting Caspases 3 and 9 activities. From the assayed anthocyanins again delphinidin was the most effective followed by cyanidin and pelargonidin, all of them 3-glucosides [17]. Anthocyanins interfere with the PI3K/Akt signalling pathway by decreasing Akt dephosphorylation induced by peroxynitrite, counteracting the effect of this species on the inactivation of this pathway. In this case, anthocyanins are showing a maximum of the effect at 4 h.

Interaction with microbiota

Finally and only as an example of the many ways in which anthocyanins can affect or prevent conditions which include an inflammatory component we and others have studied the effect of anthocyanins on the microbiota. We have studied the effect of anthocyanins on human microbiota by using an in vitro model showing that red grape anthocyanins, and more concretely malvidin-3-glucoside, can regulate specific bacteria present in human faeces [1,11]. In the same line of research Jacobsdottir and co-workers have shown that raspberry is more effective in increasing the diversity of faecal microbiota than blackberries [13].

To summarize: pelargonidin, the anthocyanidin monohydroxylated in the B-ring of the flavonoic skeleton, is more efficient in inhibiting proteasome activity (which can also produce an inhibition of NF-κB pathway). Cyanidin, the dihydroxylated one, is especially potent in inducing ABCA1/PPARγ and Nrf2 pathways and delphinidin, the trihydroxylated, is more active in down regulating NF-κB and PI3K/AKT, and it is also the most effective in neutralizing ROS. But in this very case it seems that what they all are able to do is to protect the cell from external aggressions inducing cell survival, either via inhibition of pro-inflammatory pathways or by inducing protective and antioxidant pathways (Fig. 6).

Conclusions

Not that many studies have been done on the molecular mechanisms involved in anthocyanins effect on cardiovascular and neurological health. There is a need of more studies with pure compounds in order to confirm some aspects regarding those mechanisms and the effect on them of different changes on the anthocyanidin structure.

Until now, several studies have used anthocyanidins, the aglycons, as standard molecules for those studies. However, the presence of the aglycons *in vivo* has not been confirmed so far. As far as we know anthocyanins are mainly present in animal or human plasma and tissues in their glycosylated form, glucuronidated and/or sulphated. Additionally the study of breakdown metabolites derived from anthocyanins seems to be the most promising research line in this area.
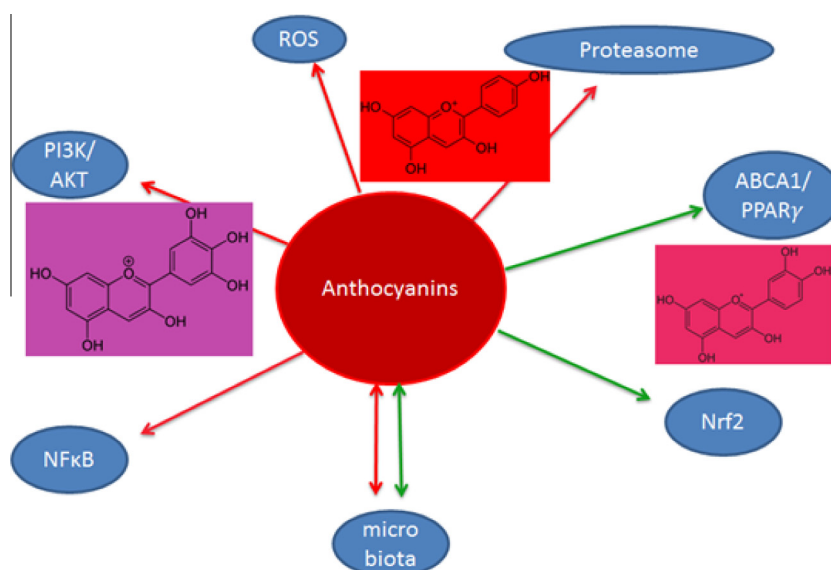

Fig. 6. Main pathways involved in the protective effect of anthocyanins against cardiovascular and neurodegenerative diseases.

When working with plant foods or plant food extracts, interpretation of results gets really complicated. If so the extracts need to be well characterized, including composition, but also, a clear identification of the plant product including not only species but variety and time and region of recollection of the plant product.

Finally, when aiming to the production of new food products with a specific target population or condition, or designing human trials with specific anthocyanin rich products, the mechanistic differences between anthocyanins should be kept in mind.

Acknowledgments

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