The 3rd International Immunonutrition Workshop was held at Platja D'Aro, Girona, Spain on 21–24 October 2009

3rd International Immunonutrition Workshop

Session 1: Antioxidants and the immune system Polyphenols from red wine are potent modulators of innate and adaptive immune responsiveness

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It is well known that the consumption of dietary polyphenols leads to beneficial effects for human health as in the case of prevention and/or attenuation of cardiovascular, inflammatory, neurodegenerative and neoplastic diseases. This review summarizes the role of polyphenols from red wine in the immune function. In particular, using healthy human peripheral blood mononuclear cells, we have demonstrated the *in vitro* ability of Negroamaro, an Italian red wine, to induce the release of nitric oxide and both pro-inflammatory and anti-inflammatory cytokines, thus leading to the maintenance of the immmune homeostasis in the host. All these effects were abrogated by deprivation of polyphenols from red wine samples. We have also provided evidence that Negromaro polyphenols are able to activate extracellular regulated kinase and p38 kinase and switch off the NF-κB pathway via an increased expression with time of the IκBα phosphorylated form. These mechanisms may represent key molecular events leading to inhibition of the pro-inflammatory cascade and atherogenesis. In conclusion, according to the current literature and our own data, moderate consumption of red wine seems to be protective for the host in the prevention of several diseases, even including aged-related diseases by virtue of its immunomodulating properties.

Flavonoids: Immune response: Polyphenols: Resveratrol

Introduction

Red wine contains a plethora of bioactive compounds, including the so-called polyphenols. These include flavonols, such as myricetin, kaempferol and the predominant quercetin, the flavan-3-ol monomers catechin and epicatechin, the oligomeric and polymeric flavan-3-ols or proanthocyanidins, many anthocyanins and a variety of phenolic acids (gallic acid, caftaric acid, caffeic acid and *p*-coumaric acid), plus the stilbene resveratrol (RSV)[1].

Once ingested dietary polyphenols are able to interact and influence the function of many biological systems in the host, even including intestinal and systemic immunity. As far as immune responsiveness is concerned, it relies on a very intricate functional network between antigen presenting cells (macrophages and dendritic cells) and T helper-1, -2, -17 and T regulatory cells, which ultimately leads to immunity or tolerance[2–13]. Furthermore, macrophages, dendritic cells and non-immune cells (endothelial and smooth muscle cells) bear, on their membrane, Toll-like receptors implicated in the activation of the pro-inflammatory pathway when triggered by exogenous or endogenous stimuli[14–29]. Any alteration of this delicate balance may account for disease outcome, even including age-related diseases, characterized by a low-grade inflammation[30].

On these grounds, the aim of this review was to clarify the main mechanisms involved in the immunomodulating ability of polyphenols (flavonoids and RSV) in health and disease.

Polyphenols

Alcohol consumption has controversial effects on human health. There is evidence that chronic alcohol abuse leads

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to cancer development (e.g. carcinoma of the liver and pancreas), damage to the brain[31] and impaired function of the immune system. On the other hand, according to epidemiological studies, it appears that light to moderate consumption of alcohol may reduce mortality. In particular, red wine consumption reduces CVD risk[32] and the protective effect is attained with two glasses of wine per day[33]. These effects are greater for red wine compared with white wine, beer, and most spirits where the amount of polyphenols is negligible[32]. In particular, polyphenols are absent from white wine because the skins, seeds and stems are present during the fermentation of red wine only, which is one of the richest sources of polyphenols in the human diet.

The potential association between red wine consumption and risk of CVD mortality has been highlighted by the 'French Paradox'[34], which refers to the finding that people in France suffer from a relatively low incidence of coronary heart disease, despite their diet being rich in saturated fats.

The effects of flavonoids and RSV on human health will be discussed below.

Flavonoids

Flavonoids have been the object of intensive investigation for a number of reasons: (i) Based on their abundance in fruits and vegetables, these substances may explain some of the health-promoting effects of these foodstuffs; (ii) some foods are particularly rich in flavonoids, such as red wine, tea, onions, apples or dark chocolate, whose favorable cardiovascular effects are well known; (iii) medicinal plants rich in flavonoids or their extracts (e.g. an extract of *Ginkgo biloba* leaves) are used within alternative as well as academic medicine; (iv) specific flavonoids (e.g. quercetin) are also available in certain countries as nutraceuticals[35].

As far as the effects of flavonoids on the immune system are concerned, primary macrophages have been studied as potential cellular targets of these substances[36]. A number of flavonoids inhibit macrophage proliferation (but not cell viability) and some additionally reduce TNFα and inducible nitric oxide synthase (iNOS) expression, probably by interfering with the NF-κB pathway. The structural determinants of activity include the C2–C3 double bond, the catechol group in the B ring and the 2-position of the B ring. Most of these flavonoids are glycosides, which are known to be hydrolyzed by bacterial enzymes in the gut. Since luteolin and quercetin are not active *in vivo* and aglycone flavonoids are absorbed in the small intestine, it is likely that glycosides act as prodrugs, releasing the biologically active aglycone in the lumen and preventing their premature absorption, which has been proven in the case of quercetin[37].

Over recent years, our group has contributed to the knowledge of flavonoid mechanisms of action in an *in vitro* model using healthy human peripheral blood mononuclear cells (PBMC). In preliminary studies, three different types of Italian red wine (Primitivo, Lambrusco and Negroamaro) were screened for modulating nitric oxide (NO) production from monocytes. The effects of flavonoids were evaluated in an indirect manner by using wine samples that have previously been deprived of these substances. NO production was maximally obtained in the presence of Negroamaro and this datum was also confirmed by iNOS expression, as assessed by Western blot. In flavonoid-deprived samples, both activities (NO release and iNOS expression) were present at negligible levels. The same was true in samples containing only ethanol[38]. The evidence that Negroamaro is the strongest inducer of NO seems to suggest a different composition of the polyphenols present in this wine, as also reported in other studies[39,40], which have related the different vasodilating ability of red wine to the type of grape and country of origin. The above results represent the first evidence of the ability of red wine to generate NO from human monocytes, despite previous reports about the endothelial origin of NO as a regulator of vascular homeostasis[41]. According to our working hypothesis[42], in response to acute consumption of flavonoids in red wine, gut-associated macrophages can generate NO that, in turn, reaches the blood via the lymphatic route. As an alternative, flavonoids that are absorbed at the intestinal level and released into the circulation are able to stimulate monocytes to produce NO. In fact, there is evidence that in healthy volunteers, anthocyanins from red grape juice are absorbed at the intestinal level[43]. In synthesis, it appears that moderate intake of red wine via NO release can prevent atherogenesis by virtue of its vasodilatory capacities[38]. As far as release of cytokines from PBMC stimulated with Negroamaro is concerned, this wine was able to skew the immune response toward the T helper-1 cell type via the release of IL-12 and consequentially of interferon (IFN)-γ[44]. Furthermore, production of IL-1β and IL-6 occurred, thus suggesting a switch toward the inflammatory pathway. On the other hand, release of the anti-inflammatory cytokine IL-10 was also triggered by Negroamaro, thus indicating that the production of IL-6 and IL-1β was still under the control of IL-10[45]. In turn, IL-6 stimulates IL-10 production, generating a local feedback loop in order to limit the proinflammatory pathway[46]. This event accounts for the maintenance of the inflammatory:anti-inflammatory ratio.

As far as B cell responses are concerned, in our test system flavonoids favoured the production of both IgA (mucosal response) and IgG (systemic response). In particular, at the gut-associated lymphoid tissue level, one can speculate that flavonoid-activated B cells may migrate to distant organs, such as the spleen and the secretory glands in the body[42], thus representing an amplification loop of the immune response. These immunological mechanisms are able to protect the host against pathogens as well as against neoplastic events via cytotoxic T cell and natural killer cell activation[47,48]. Based on these concepts, moderate red wine assumption might be useful to consider in age-related disorders because it is known that immunosenescence is characterized by a status of dysregulated immunity that leads to the increased susceptibility of the elderly to infections and autoimmune disease[49,50]. In this framework, it is worth mentioning that macrophages from old individuals produce much less hydrogen peroxide and NO than young individuals[51]. Interestingly, it has been reported that aged mice express less total p38 and C-Jun

N-terminal kinase[52] and, at the same time, an elevation of p38 and extracellular regulated kinase in red-wine-treated PBMC was observed[53]. Therefore, the increased expression of p38 by flavonoids[54] may trigger the induction of the IFN-γ pathway, increase the release of pro-inflammatory cytokines and promote Ig class switch in B cells, since p38 is a common denominator for all these pathways[55]. In conclusion, moderate red wine consumption or intake of flavonoids can be advantageous in the elderly via increased production of NO by macrophages and p38-induced IFN-γ release.

In our experiments, we found that the expression of phosphorylated p38 was enhanced by lipopolysaccharide (LPS) alone, while in samples simultaneously stimulated with LPS and Negroamaro, a decreased expression of activated p38 was observed. This may indicate that flavonoids tend to down-regulate the LPS-induced p38 phosphorylation pathway[42]. Furthermore, this fact may be beneficial in the course of Gram-negative infections, where inhibition of p38α activity by the inhibitor SB203580 reduced mortality related to LPS-dependent shock and occurrence of collagen-induced arthritis[56,57]. These findings have recently been confirmed by the demonstration that macrophage deletion of p38α mitigates noxious effects triggered by LPS[58].

Interestingly, p38 *per se* may be responsible for NO release and iNOS expression, probably through another nuclear factor, such as signal transducer and activator of transcription-1 (Stat-1) or activator protein-1. In other experiments, we reported an elevated production of IFN-γ by monocytes treated with flavonoids[44]. In this context, IFN-γ could act together with p38 as an activation signal of Stat-1[59,60] that, in turn, by binding to motifs at $-5 \cdot 2$ and $-5 \cdot 8$ kb in the iNOS promoter, might determine iNOS expression and, therefore, NO synthesis.

The NF-κB family of transcription factors consists of five members of the mammalian NF-κB family: p50/p105, p52/p100, p65, c-Rel and RelB, which are kept inactive in the cytoplasm by association with inhibitors (I)κB proteins[61] identified as IκBα, IκBβ and IκBε. They maintain NF-κB dimers in the cytoplasm and are crucial to signal responsiveness. IκBα is rapidly degraded during activation, of canonical NF-κB signalling pathways, leading to the release of multiple NF-κB dimers. Binding to IκBα prevents the translocation of NF-κB into the nucleus, thereby maintaining NF-κB in an inactive state. In response to an inflammatory stimulus, cytokines or viral infections, IκB proteins are rapidly degraded by the multicatalytic proteasome[62]. IκBα has the capacity to both prevent NF-κB binding and dissociate NF-κB from specific DNA consensus sequences. Nuclear localization of IκBα is induced by stimuli activating NF-κB and can be considered as part of a physiological mechanism regulating NF-κB-dependent transcription[63].

The main observation related to IκBα is that this factor enters the nucleus steadily in cells continuously exposed to stimulation, but is constantly degraded in the nuclear compartment as long as stimulation persists[63]. In our previous study[53], the phosphorylated form of IκBα was expressed at lower levels in PBMC treated with Negroamaro compared with the unphosphorylated form. However, there is

Table 1. The salient effects of Negroamaro flavonoids on human healthy mononuclear cells

- release of NO from human monocytes;
- induction of iNOS expression;
- polarization of the T helper-1 response;
- balance of the inflammatory/anti-inflammatory ratio;
- production of IgG and IgA;
- increased expression of phosphorylated p38 and ERK;
- switch-off of the NF-κB pathway via an increased expression with time of the IκBα phosphorylated form.

NO, nitric oxide, NOS, inducible NO synthase; ERK, extracellular regulated kinase.

evidence that more molecules of IκBα are generated with time and, therefore, this molecule participates in a feedback loop, where newly synthesized IκBα inhibits the activity of NF-κB[64]. These data suggest that flavonoids switch off the NF-κB pathway, thus reducing the release of pro-inflammatory cytokines (e.g. IL-1β).

In another series of results obtained by immunofluorescence analysis, we demonstrated that cells underwent a marked expression of p65 after treatment with whole wine and flavonoids alone, although this was lower than that observed with LPS-treated PBMC[53]. This finding reinforces the concept of the beneficial effects of flavonoids on human health, since an excessive production of p65/NF-κB would lead to induction of several genes that ultimately generate noxious effects into the host[53]. In conclusion, molecular mechanisms elicited by flavonoids on human PBMC seem to play a beneficial role in the host. One can postulate that NO release following activation of extracellular regulated kinase and p38 kinase is able to inhibit platelet aggregation, thus reducing the influx of atherogenic monocytes and LDL through endothelial cells into the wall of arteries and ultimately limiting atherogenesis. Furthermore, NO production may reduce infectious events, such as *Chlamydia pneumoniae* infection, which represents an important etiological factor in atherosclerosis[65,66].

As far as the role of LPS in atherogenesis is concerned, inhibition of phosphorylated p38 expression in the presence of LPS, as well as the regulatory role of IκBα mediated by flavonoids, should be taken into consideration in the arrest of the proinflammatory cytokine pathway in a variety of diseases.

In Table 1, the major effects of flavonoids from Negroamaro wine are listed.

Resveratrol

RSV (3,4′,5-trihydroxystilbene) is contained in the skins of red fruits, such as mulberries and red grapes[67,68], and therefore its intake occurs along with flavonoids.

Over recent years, increasing interest in RSV from red wine in relation to human health has been reported. In fact, RSV prevents or delays the onset of age-related diseases, such as diabetes, inflammation, Alzheimer's disease and CVD, also inducing neuroprotection and inhibiting neoplastic growth[69–77]. The therapeutic potential of RSV has

Table 2. Effects of RSV on different systems

- increased GLUT-4 expression and reduced endothelin expression and cardiac apoptosis in a genetic model of type-2 diabetes[87];
- increased insulin sensitivity caused by a decrease of the blood glucose level in mice fed a high-energy diet[88];
- attenuation of diabetic nephropathy in rats[89];
- protection of the brain from traumatic injury[90];
- inhibition of excitatory synaptic transmission in the rat hippocampus[91,92];
- neuroprotection mediated by decreased oxidative stress and increased NO release[93].

been demonstrated by the extended lifespan and ameliorated motor function in mice fed a high-energy diet[78]. The potential benefits to human health include the full range of CVD-associated conditions, e.g. myocardial infarction, arrhythmias, hypertension, hypertrophy, inflammation leading to fibrosis, atherosclerosis and thrombosis.

Although RSV acts as an antioxidant, primarily by increasing NO bioavailability, it can also exhibit pro-oxidant properties in the presence of transition metal ions such as Cu, thus leading to oxidative breakage of cellular DNA[79], which seems to be the common denominator for anticancer and chemopreventive properties of the polyphenol family[80]. In fact, plant polyphenols are able to modulate the effects of deregulated cell cycle checkpoints, as a basis for cancer chemoprevention[81].

Low-grade inflammation is implicated in various diseases such as the metabolic syndrome[82], rheumatoid arthritis and neurodegenerative disorders[83]. The cardioprotection and cancer prevention of RSV may depend on its anti-inflammatory effects, such as the inhibition of synthesis and release of pro-inflammatory cytokines and of eicosanoids and the inhibition of immune cell activation. Enzyme inhibition of cyclooxygenase-1 or cyclooxygenase-2 by the inhibitory effects of RSV on transcription factors such as NF-κB or activator protein-1 has also been reported[84,85]. Furthermore, RSV associated with moderate wine intake activates platelet endothelial NOS and, in this way, blunts the pro-inflammatory pathway linked to p38 mitogen-activated protein kinase, thus inhibiting reactive oxygen species production and ultimately platelet function. In another study, RSV[86] was able to reduce oxidative burst, chemotaxis and degranulation in human neutrophils as well as pro-inflammatory cytokines, such as IL-6, IL-1β, TNF-α and chemokine macrophage inflammatory protein-1α *in vitro*, as well as in a murine model of acute peritonitis. In this regard, two important targets of RSV were identified, namely the lipid mediator phospholipase D and the proinflammatory kinase SphK.

Other effects of RSV are listed in Table 2.

Conclusion and future perspective

Current nutritional approaches to prevent inflammatory, metabolic, neoplastic and aged related-diseases are a major

strategy for the improvement of human health and longevity[94].

In this review, data from the current literature and our own data demonstrate the ability of red wine to modulate the immune response by virtue of its flavonoid content and RSV. As a result, low-grade inflammation, which is a common denominator of several diseases, can be prevented or mitigated. In fact, in terms of clinical applications, other diseases besides atherosclerosis can be prevented by wine consumption. For instance, in the Bordeaux region, a negative correlation was found between moderate wine intake and Alzheimer's disease. Furthermore, in the third Copenhagen City Heart Study on aged people, monthly and weekly consumption of wine was associated with a lower risk of dementia[95]. In a previous study, based on a cohort of 1367 subjects with dementia, flavonoid ingestion was inversely correlated to the risk of dementia[96]. In another study, a protective effect of wine consumption on age-related macular degeneration compared to non-drinkers was reported[97,98].

In conclusion, polyphenols from red grape could be considered along with other dietary substances such as pre/probiotics and oligoelements (e.g. Zn and Se) as potentiators of the immune response.

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