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The Influence of Wine Polyphenols on Reactive Oxygen and Nitrogen Species Production by Murine Macrophages RAW 264.7

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Summary

The aim was to study the antioxidant properties of four wine polyphenols (flavonoids catechin, epicatechin, and quercetin, and hydroxystilbene resveratrol). All three flavonoids exerted significant and dose-dependent scavenging effects against peroxyl radical and nitric oxide in chemical systems. The scavenging effect of resveratrol was significantly lower. All polyphenols decreased production of reactive oxygen species (ROS) by RAW264.7 macrophages. Only quercetin quenched ROS produced by lipopolysaccharide-stimulated RAW264.7 macrophages incubated for 24 h with polyphenols. Quercetin and resveratrol decreased the release of nitric oxide by these cells in a dose-dependent manner which corresponded to a decrease in iNOS expression in the case of quercetin. In conclusion, the higher number of hydroxyl substituents is an important structural feature of flavonoids in respect to their scavenging activity against ROS and nitric oxide, while C-2,3 double bond (present in quercetin and resveratrol) might be important for inhibition of ROS and nitric oxide production by RAW 264.7 macrophages.

Key words

Antioxidants • Polyphenols • Macrophages • Oxidative stress • Nitric oxide

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Introduction

The overproduction of reactive oxygen and nitrogen species (ROS and RNS, respectively) by phagocytes causes oxidative damage to membrane lipids, DNA, proteins and lipoproteins. These reactions have functional consequences, which may be deleterious to cells and tissues. Thus, the inhibition of ROS and RNS production is a popular target for the attenuation of many inflammatory diseases (Shen *et al.* 2002).

Dietary polyphenols with antioxidative effects from fruit and vegetables play an important role in a prevention of the oxidative stress (Mojžišová and Kuchta 2001, Osawa 1999). Another important source of polyphenolic antioxidants is wine, particularly red wine. It has been demonstrated that polyphenols from wine have not only antioxidative but also anti-inflammatory effects (Oak *et al.* 2005) and that they can prevent cardiovascular diseases (Babal *et al.* 2006). It is furthermore suggested that they prevent free radical-mediated lipid peroxidation of low density lipoproteins (LDL), which is associated with cell aging and chronic disesases such as atherosclerosis (Dell'Agli *et al.* 2004, Cook and Samman 1996, Oak *et al.* 2005, Rajdl *et al.* 2006). It is postulated that the antioxidant and free radical scavenging properties of phenolic compounds, present in red wine, may partly explain the ”French paradox”, i.e. the fact that French people have low incidence of coronary heart disease, despite having a diet high in fat and being heavy smokers (Aruoma 1994). The main polyphenolic compounds in red wine belong to two major classes: flavonoids and stilbenes. Of the flavonoids, (+)-catechin, (–)-epicatechin and quercetin and of the stilbenes, trans-resveratrol, are the most abundant polyphenols in wine. Exact mechanisms by which flavonoids protect against oxidative stress-mediated diseases (such as atherosclerosis) are still a matter of debate (Benito *et al.* 2004).

The aim of this experiment was to study the antioxidant properties of four wine polyphenols (catechin

– CAT, epicatechin – EPI, quercetin – QUE, resveratrol – RES) against peroxyl radical and nitric oxide, their immediate and long-term effects on the production of reactive oxygen and nitrogen species by RAW 264.7 macrophages, and their influence on the expression of inducible nitric oxide synthase. Special attention was paid to differentiate between scavenging and inhibiting activity of the studied polyphenols with respect to their chemical structure. As far as we know, there are no similar investigations on the same selection of polyphenols, especially concerning the effects of polyphenols on various parameters linked to the generation of nitric oxide. The advantage of the study is that it took into consideration many factors: kind and dose of polyphenols, time of their action, and different reactive metabolites.

Methods

Materials

Murine RAW 264.7 macrophage cell line was obtained from the American Type Culture Collection (ATCC, Manassas, Virginia, USA). Cells were maintained in a Dulbecco`s Eagle medium (DMEM) (Sigma-Aldrich, St. Louis, Missouri, USA) supplemented with a 10 % fetal bovine serum, gentamycin (0.045 mg/l), glucose (3.5 g/l) and $NaHCO_3$ (1.5 g/l). The stock solution of lipopolysaccharide (LPS) from *Escherichia coli* serotype 0111:B4 (Sigma-Aldrich, St. Louis, Missouri, USA) was made up at 10^{-3} g/l in Hanks' balanced salt solution without phenol red (HBSS, pH 7.4). The final concentration of LPS in a reaction mixture was 10^{-7} g/l. Polyphenols (CAT, EPI, QUE and RES) were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Stock solutions ($2x10^{-2}$ mol/l) were always prepared fresh, in 99.8 % ethanol. Then, concentrations of $2x10^{-4}$, $5x10^{-4}$, $1x10^{-3}$, $2x10^{-3}$ mol/l were prepared in RPMI 1640 and added to the reaction mixture to obtain final concentrations of 10^{-5}, $2.5x10^{-5}$, $5x10^{-5}$ and 10^{-4} mol/l, respectively.

ABAP [2,2-azo-bis(2-amidinopropane) hydrochloride] was purchased from Polyscience (Warrington, Pennsylvania, USA) and Trolox (6-hydroxy-2,5,7,8,-tetramethyl-chroman-2-carboxylic acid) from Sigma-Aldrich (St. Louis, Missouri, USA). The stock solution of 10^{-2} mol/l luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) (Molecular Probes, Eugene, Oregon, USA) was prepared in 0.2 mol/l sodium borate buffer, pH 9.0 (1.24 g of H_3BO_3 and 7.63 g of $Na_2B_4O_7 \cdot 10H_2O$ in one liter of redistilled water). All other reagents were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). PMA (phorbol 12-myristate 13-acetate) was dissolved in dimethylsulphoxide to obtain $3x10^{-3}$ mol/l stock solution.

Total radical-trapping antioxidant parameter (TRAP)

The luminol-enhanced chemiluminescence (CL) assay for TRAP was measured using a Luminometer 1251 (BioOrbit, Turku, Finland). The method is based on the measurement of peroxyl radicals produced at a constant rate by a thermal decomposition of ABAP. The TRAP value is determined from the time period during which the CL signal is diminished by antioxidants (Slavíková *et al.* 1998). Trolox, a water-soluble analogue of α-tocopherol, was used as a reference inhibitor for the calculation of the TRAP value.

The reaction mixture contained 475 µl of sodium phosphate buffered saline (PBS 10^{-1} mol/l, pH 7.4), 50 µl of 10^{-2} mol/l luminol in 10^{-1} mol/l borate buffer (pH 10.0), and 20 µl of examined compound. The cuvettes were incubated at 37 °C in the temperature-controlled carousel of the luminometer for 10 min. Then, 50 µl of $4x10^{-1}$ mol/l ABAP (prepared in 10^{-1} mol/l PBS) was added. TRAP value for the sample was obtained from the equation: $TRAP = n\,[Trolox]\tau_{sample} / f\,\tau_{Trolox}$, where n is the stoichiometric factor of Trolox (2.0), τ is the time period of diminished chemiluminescence, and *f* is the dilution factor of the sample.

Scavenging of nitric oxide

Tested compounds were diluted to reach final concentrations of 10^{-5} – 10^{-4} mol/l in a final volume of 10 ml of PBS. 1 µl of saturated nitric oxide solution was added and the concentration of nitric oxide was measured amperometrically using the ISO-NO Mark II isolated nitric oxide meter and ISO-NOP sensor (both from WPI, Sarasota, FL, USA). The method is based on a diffusion of NO through a selective membrane covering the sensor and its oxidation at the working electrode, resulting in an electrical current. The scavenging effect of tested compounds was evaluated based on a decrease in NO concentration in reaction mixture as compared to the control. The data are expressed as a redox current in pA.

Experimental design

When the immediate effect of polyphenols was studied, 2 x 10^5 of RAW 264.7 cells in 100 µl of DMEM were allowed to adhere for 60 min to the edge of each of

the 96-well plate and then all chemicals were added immediately before CL was measured.

When the long-term effect was studied, 96-well cultivation plates ($2x10^5$ cells per well) and 6-well cultivation plates ($2x10^6$ cells per well) were used for CL measurement and Western-blot analysis (and Griess reaction), respectively. Adhered cells (1 h) were preincubated with one of the polyphenols at an indicated concentration for 1 h prior to the 24 h of incubation with LPS (10^{-4} g/l). Adherence as well as incubation steps proceeded at 37 °C with 5 % CO_2. After 24 h the 96-wells were gently washed with HBSS and CL was measured (as described below). The supernatants of the 6-well plates were removed and used for the determination of nitrites by Griess reaction as described below. Cells were used for a Western-blot analysis as described below.

Chemiluminescence assay

The luminol-enhanced CL of RAW 264.7 macrophages was measured using microtitre plate Luminometer LM-01T (Immunotech, Prague, Czech Republic) as described previously (Lojek *et al.* 1997). The principle of the method is based on a luminol interaction with the phagocyte-derived ROS, which results in large measurable amounts of light. Each well (in 96-well culture plates) contained $2x10^5$ RAW 264.7 cells, luminol (at a final concentration of 10^{-3} mol/l) and PMA (at a final concentration of $8x10^{-7}$ mol/l), which was selected on the basis of previous results (Lojek *et al.* 1997) and one of the polyphenols at final concentrations of 0, 10^{-5}, $2.5x10^{-5}$, $5x10^{-5}$ and 10^{-4} mol/l. The total reaction volume of 250 µl was adjusted with HBSS. The assays were run in duplicates. Light emission expressed as relative light units (RLU) was recorded continuously at 37 °C for 60 min. Intensity of the CL reaction is expressed as the integral of the obtained kinetic curves which correspond to the total amount of light produced during the time of measurements.

Determination of nitric oxide

The production of nitric oxide (NO) was estimated indirectly as the accumulation of nitrites (NO_2^-), the metabolic end-product of NO metabolism, in the medium using the Griess reagent as described previously (Migliorini *et al.* 1991). Sodium nitrite was used as a standard. 150 µl of culture supernatant was added to 150 µl of Griess reagent (Sigma-Aldrich, St. Louis, Missouri, USA), then incubated for 15 min in a dark at room temperature and absorbance was measured at 532 nm on a SLT Rainbow spectrophotometer (Tecan, Crailsheim, Germany).

Detection of inducible nitric oxide synthase (iNOS) by Western blot

Cells were washed with cold PBS, scraped and then lysed in the lysis buffer (1 % sodium dodecyl sulphate – SDS, 10^{-1} mol/l Tris pH 7.4, 10 % glycerol, 10^{-3} mol/l sodium ortho-vanadate, 10^{-3} mol/l phenyl-methanesulfonyl fluoride). Protein concentrations were measured with the DC protein assay (Bio-Rad, Hercules, California, USA) using bovine serum albumin as a standard. Equal amounts of proteins (20 µg) were applied on SDS-polyacrylamide gel electrophoresis. Proteins were electrically transferred from the gel to a nitrocelulose membrane and immunoblotted with rabbit antiserum against the murine iNOS (Transduction Lab, Lexington, Kentucky, USA). Horseradish peroxidase-conjugated with anti-rabbit IgG antibody was used as a secondary antibody. The blots were visualized using ECL+ kit (Amersham, Arlington Heights, Illinois, USA) and exposed to CP-B X-ray films (Agfa, Brno, Czech Republic).

Viability

Viability was set using ATP kit SL (BioThema AB, Hanince, Sweden) on microtitre plate Luminometer LM-01T (Immunotech, Prague, Czech Republic).

Statistical analysis

All experiments were done in duplicates and repeated six times. All data are reported as means ± S.E.M. The data were analyzed using the non-parametric Wilcoxon test or one-way analysis of variance (ANOVA) using Statistica for windows 5.0 (Statsoft, USA). $P \leq 0.01$ value was considered to be significant.

Results

Immediate effect of polyphenols

The total antioxidant capacity of four polyphenols studied (CAT, EPI, QUE and RES) measured as the ability to scavenge chemicaly generated peroxyl radical is shown in Figure 1. All polyphenols showed a significant antioxidant capacity, which increased in a dose-dependent manner. Flavonoids (CAT, EPI, QUE) were observed to show an approximately fourfold higher antioxidant capacity than hydroxystilbene RES.

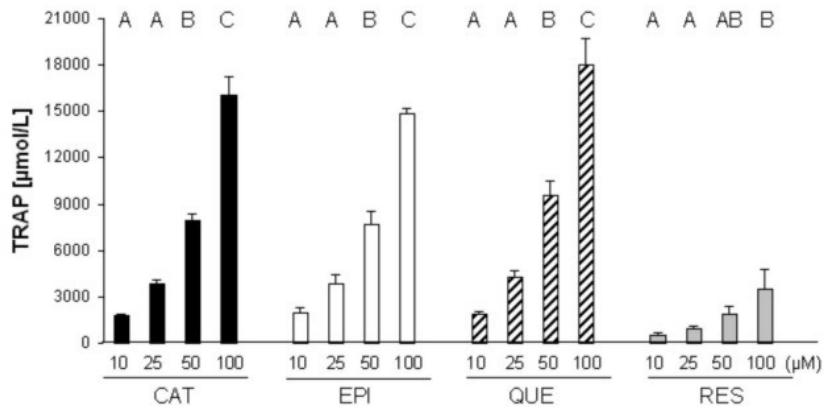

Fig. 1. Total antioxidant capacity of polyphenols. TRAP is expressed as µmol of the peroxyl radical trapped per one liter of polyphenolic solution. The changes of the parameter were found to be significant at the level of p = 0.01 using ANOVA test. Statistically significant contrasts between the values are marked by different capital letters.

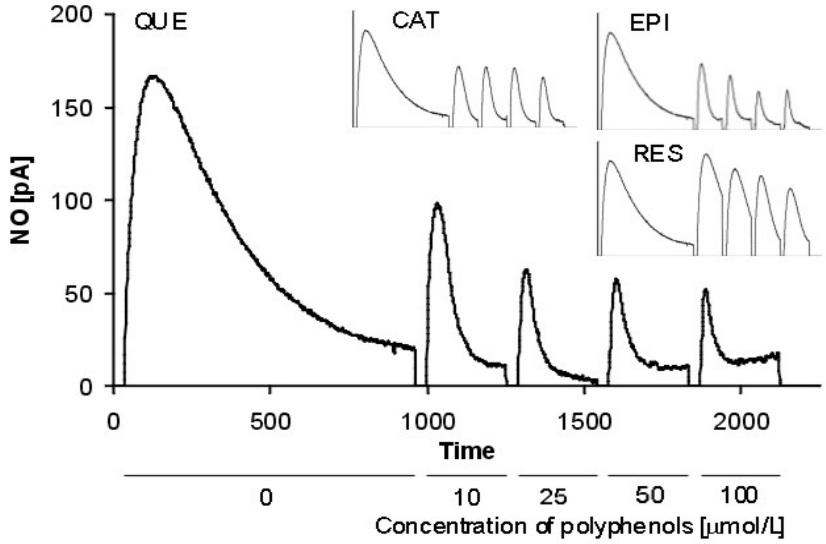

Fig. 2. The ability of polyphenols to scavenge NO determined ampero-metrically in a chemical system. The concentration of NO is expressed as a redox current in picoampers (pA).

Similar results were obtained when the ability of tested compounds to scavenge nitric oxide was evaluated. Flavonoids (CAT, EPI, and especially QUE) were observed to markedly scavenge NO. However, resveratrol scavenged NO only mildly in the highest tested concentration (Fig. 2).

The ability of polyphenols to reduce the oxidative stress caused by biologically generated radicals was studied using murine macrophages RAW 264.7. All polyphenols dose-dependently inhibited the chemiluminescence produced by the PMA-stimulated RAW 264.7 (Fig. 3). The effect of RES was the highest among all compounds tested, because even the lowest concentration markedly inhibited chemiluminescence. Thus, RES seems to be a more potent inhibitor of the oxidative burst than flavonoids.

Long-term effect of polyphenols

LPS (10^{-4} g/l) increased the ROS production by 190 % (51.2 x 10^4 vs. 17.6 x 10^4 RLU in untreated controls), as measured by PMA-activated CL. CAT, EPI and RES did not have any inhibitory effect on ROS production (data not shown). Conversely, QUE significantly decreased the ROS production of LPS-stimulated RAW 264.7 in a dose-dependent manner (Fig. 4A).

LPS evoked a 30-fold induction of nitrite production as opposed to the untreated control ($34.4x10^{-6}$ vs. $1.1x10^{-6}$ mol/l NO_2^-/$2x10^6$ cells, respectively). This induction was inhibited by QUE ($2.5x10^{-5}$-10^{-4} mol/l) treatment in a dose-dependent manner (Fig. 4B). RES at concentrations of $5x10^{-5}$ and 10^{-4} mol/l cut the NO production by 7 and 26 %, respectively. However, no significant inhibition by CAT and EPI was found (data not shown).

The cytotoxicity of polyphenols in RAW 264.7 cells was examined by luminometrical detection of ATP concentration. None of the polyphenols affected the

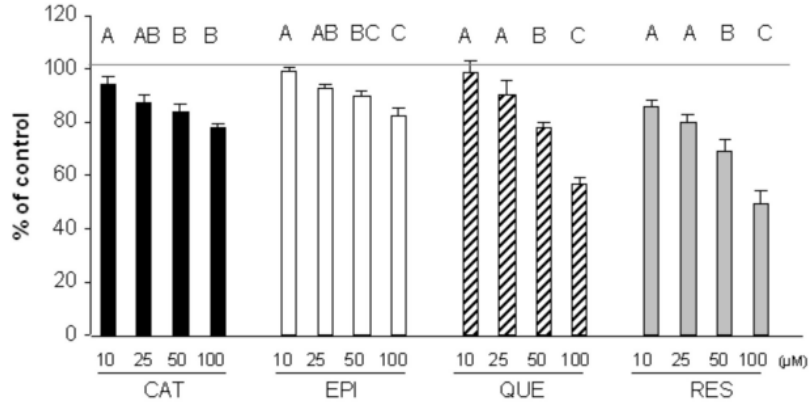

Fig. 3. Activity of polyphenols to scavenge reactive metabolites produced by RAW 264.7 cells (measured by chemiluminiscence). The changes of the parameter were found to be significant at the level of $p \leq 0.01$ using Wilcoxon test. Statistically significant difference between the values are marked by different capital letters.

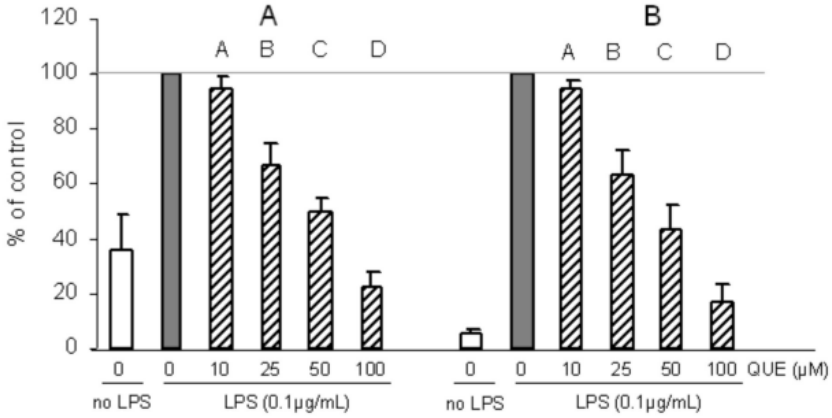

Fig. 4. The effect of quercetin (QUE) on chemiluminescence activity and nitrite production by RAW 264.7 cells. Cells were pretreated with the indicated concentrations of QUE for 1 h before being incubated with LPS (10^{-4} g/l) for 24 h. Control cells were incubated with vehicle alone. The changes of the parameter were found to be significant at the level of $p \leq 0.01$ using Wilcoxon test. Statistically significant contrasts between the values are marked by different capital letters. (A) Inhibition of reactive metabolite production by QUE in LPS-stimulated RAW 264.7 macrophages. Before measurement, supernatant was removed and cells were washed twice with PBS. Phorbol-myristate acetate was used to activate the oxidative burst of RAW 264.7 measured by chemiluminiscence. (B) Inhibition of nitrite production by QUE in LPS-stimulated RAW 264.7 cells. The cultured supernatants were subsequently isolated and analyzed for nitrite production.

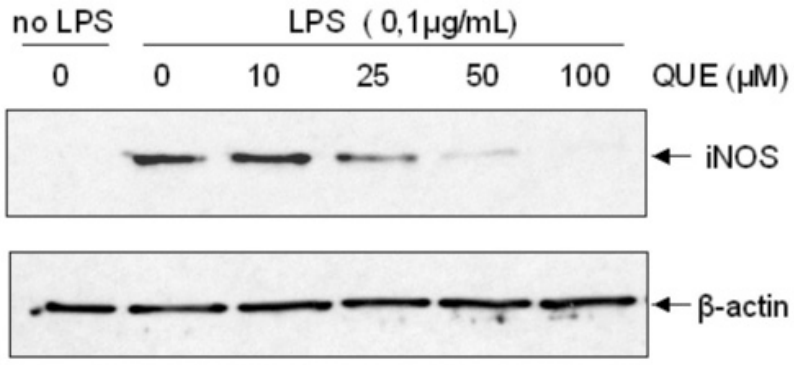

Fig. 5. Inhibition of iNOS protein expression by QUE. Cultures were set up as described in the legend to Fig. 4. Equal loading of proteins was verified by β-actin immunoblotting. One of three representative experiments is shown.

viability of RAW 264.7 cells (data not shown).

In view of the involvement of iNOS in the inflammatory process, we monitored iNOS protein expression by Western blot in RAW 264.7 exposed to polyphenols. As shown in Figure 5, expression of the iNOS protein was not detectable in unstimulated cells, but markedly increased 24 h after LPS (10^{-4} g/l) treatment. Treatment with QUE showed a concentration-dependent inhibition of iNOS protein expression in LPS-stimulated RAW 264.7 cells. CAT, EPI and RES did not influence iNOS expression in LPS-activated RAW 264.7 cells (data not shown).

Discussion

In our paper, four wine polyphenols (CAT, EPI,

QUE and RES) were studied from the viewpoint of their antioxidant capacity, their ability to scavenge biologically produced ROS and RNS and their influence on nitric oxide production and iNOS expression. Concentrations of polyphenols used in our experiments ranged between 10^{-4} – 10^{-5} M which corresponded to the red wine content of individual compounds: 191 mg/l (6 x 10^{-4} M) CAT, 82 mg/l (3x10^{-4} M) EPI, 8-16 mg/l (2.5-5.0 x 10^{-5} M) QUE, and 1-8 mg/l (0.5-3.5 x 10^{-5} M) RES (Pendurthi and Rao 2002). The knowledge of absorption, biodistribution and metabolism of polyphenols is partial and incomplete. Some polyphenols are bioactive compounds that are absorbed from the gut in their native or modified form. They are subsequently metabolized to products detected in the plasma and then excreted. At concentrations found *in vivo* in human plasma (low nanomolar range), the effect of polyphenols is negligible in comparison with endogenous protection mechanisms against oxidative stress (Huisman *et al*. 2004).

In agreement with other authors (Cuendet *et al.* 2000, Nakao *et al.* 1998, Scott *et al.* 1993) we observed a significant antioxidant capacity against peroxyl radical of all studied polyphenols. Some authors have even found that QUE and CAT showed a greater efficacy to scavenge peroxyl radical on a mole to mole basis than the antioxidant nutrients vitamin C, vitamin E, and beta-carotene (Rice-Evans 1995). In our study, flavonoids (CAT, EPI, QUE) showed an approximately four-fold higher ability to scavenge peroxyl radical than hydroxystilbene RES. The antioxidant potential of polyphenols depends on the number and arrangement of the hydroxyl groups and the extent of structure conjugation (Robak *et al.* 1988). They can donate hydrogen atom from their hydroxyl groups and stabilize the phenoxy radical formed by delocalization of the unpaired electron within the aromatic structure. It is well-known that aromatic compounds containing hydroxyl groups, especially those having an O-dihydroxy group on ring B, appear to be important scavengers as reported for flavonoids (Fauconneau *et al.* 1997). RES, a stilbene, is also known to have a strong antiradical activity, which is due to the presence of a conjugated double bond, which makes the electrons more delocalized (Khanduja and Bhardwaj 2003). The higher TRAP of flavonoids found in our experiments may be caused by the fact that flavonoids contain multiple hydroxyl groups (five OH groups) in comparison to RES (three OH groups), which are able to donate hydrogen atoms to peroxyl radicals and so have a greater potential to act as a scavenger of peroxyl radicals. This is also supported by the findings of López *et al.* (2003), who reported QUE to have higher antioxidant capacity against peroxyl radical than RES. On the other hand, Yilmaz and Toledo (2004) found the RES to be a more potent scavenger of chemically generated peroxide radicals than CAT and EPI.

Since cellular systems generate a variety of radicals including superoxide, hydroxyl and peroxyl radicals, nitric oxide and peroxynitrite, other experiments were performed to demonstrate the ability of flavonoids and RES to scavenge biologically generated radicals.

All tested compounds dose-dependently scavenged the free radicals that were produced by the PMA-stimulated RAW 264.7 cells. RES, interestingly, seems to be a more potent scavenger, although its TRAP was the lowest. The efficiency of individual polyphenols to scavenge peroxyl radical and inhibit the oxidative burst in macrophages was not always the same because of a different specificity of a polyphenol to scavenge peroxyl radical particularly and other free radicals involved in the process of an oxidative burst of macrophages. The TRAP test provides information on the reactivity of phenolic compounds with only one radical in a buffered solution. On the other hand, in the PMA-stimulated macrophage CL assay, a combination of many ROS is present. Moreover, the mechanisms of flavonoid actions may differ from that of stilbenes. In addition to their antioxidative properties, some polyphenols act as metal chelating agents and inhibit the superoxide-derived Fenton reaction, which is an important source of the most reactive hydroxyl radicals. Various authors consider chelation of metal ions as the main mechanism of polyphenolic action (Iwahashi 2000, Morel *et al.* 1994), while others consider ROS scavenging to dominate in the antioxidant effects (Fremont *et al.* 1999). Belguendouz *et al.* (1997) reported that RES protects LDL against peroxidative degradation mainly by chelating copper whereas flavonoids are better scavengers of free radicals. The ability to inhibit the Fenton reaction could explain the effectiveness of RES against biologically generated radicals in contrast to the system, where radicals are chemically produced in a copper-free buffered solution.

All phenomena previously described are responsible for a short-term response mediated by wine polyphenols. Recent studies have pointed out that polyphenolic compounds from several sources may also have long-term effects, as they are able to modulate gene expression in different transformed cell lines and in macrophages (Dell'Agli *et al.* 2004). We used a bacterial

LPS for the induction of inflammation processes in RAW 264.7. LPS, as an outer membrane component of bacteria, triggers the generation of reactive oxygen intermediates as well as the secretion of a variety of inflammatory mediators, such as nitric oxide. In our experiment, a 190 % increase in ROS production was observed when RAW 264.7 cells were incubated with LPS (24 h) in comparison with an untreated control. The incubation of cells with QUE diminished their LPS-activated production of ROS in a concentration-dependent manner (Fig. 4A). We found that QUE, in a concentration of 10^{-4} mol/l, reduced the ROS release even to the level of non-LPS-treated cells. Conversely, CAT, EPI and RES did not decrease the amount of ROS produced by activated macrophages. This indicates that QUE is the most effective modulator of oxidative stress in the long-term.

The large amount of NO produced in response to bacterial lipopolysaccharide plays an important role in endotoxemia and inflammatory conditions (Bellot *et al.* 1996). Therefore, drugs that inhibit NO generation by inhibiting iNOS expression or its enzyme activity may be beneficial in treating diseases caused by an overproduction of NO (Stoclet *et al.* 1998). To investigate the effect of polyphenols on NO production, we measured the accumulation of nitrite, the stable metabolite of NO, in culture media. We found a huge NO release by LPS-stimulated cells as opposed to untreated control. The results are in full agreement with the data of others (Shen *et al.* 2002, Wadsworth and Koop 1999, Wadsworth *et al.* 2001).

When LPS-activated cells were incubated with QUE or RES, significant and dose-dependent decrease of the NO production was observed after 24 h. It seems likely that QUE and RES inhibit NO production by several mechanisms. Suppression of NO release may be attributed to direct NO scavenging activity, which was previously suggested to be due to their ability to scavenge an exogenous NO donor sodium nitropruside (SNP) *in vitro* (Chan *et al.* 2000). Direct NO scavenging activity of studied flavonoids was also proved in our experiments. Another possible mechanism is a modulation of iNOS protein expression. To investigate, whether QUE and RES are able to decrease iNOS protein expression, Western blot analysis of RAW 264.7 exposed to LPS and one of these polyphenols was monitored. As shown in Figure 5, expression of the iNOS protein was not detectable in unstimulated cells, but markedly increased 24 h after LPS treatment. Treatment with QUE showed a concentration-dependent inhibition of iNOS protein expression in LPS-stimulated RAW 264.7 cells. This finding is in agreement with results of Jung and Sung (2004) who found an inhibitory effect of 5 μmol/l QUE on the expression of iNOS and COX-2 enzymes in lipopolysaccharide-activated RAW 264.7 cells. The results of Kim *et al.* (1999) indicated that the inhibitory activity of studied flavonoids was not due to direct inhibition of iNOS enzyme activity as measured by [^{3}H]citrulline formation from [^{3}H]arginine. The regulation of iNOS expression is complex, but appears to occur primarily at the level of transcription. Activation of mitogen-activated protein kinases (MAPKs) and the redox-sensitive transcription factors, nuclear factor-κB (NF-κB) and activator protein 1 (AP-1) are key events in the signal transduction pathways mediating iNOS induction in macrophages exposed to LPS (Sherman *et al.* 1993, Xie *et al.* 1994). Wadsworth *et al.* (2001) have found that QUE inhibited p38 MAPK activity and caused the inhibition of iNOS mRNA expression which resulted in decreasing iNOS protein level and NO release in LPS-stimulated RAW 264.7 macrophages. It is not clear whether QUE acts more by inhibiting iNOS expression or by direct scavenging of NO. Chan *et al.* (2000) speculate that QUE and RES may act more by scavenging of NO radicals than by inhibition of iNOS gene expression. The rationale for this deduction is that these compounds were not very effective in reducing iNOS mRNA, while they readily scavenged NO produced by SNP. In our study, inhibition of iNOS protein expression was in parallel with the comparable inhibition of NO production, thus agreeing with the results obtained by Chen *et al.* (2001), Chan *et al.* (2000) and Wadsworth *et al.* (2001). Therefore we propose that the suppression of NO by QUE was mainly mediated by inhibition of iNOS protein expression. As stated earlier, NO plays an important role in the pathogenesis of various inflammatory diseases. Therefore, the inhibitory effect of QUE on iNOS gene expression suggests that this is one of the mechanisms responsible for the anti-inflammatory action of QUE.

Although RES at higher concentrations decreased NO production, it did not influence iNOS expression in LPS-activated RAW 264.7 cells. Our results agree with those of Cho *et al.* (2002), who found that a higher concentration of RES than needed for the inhibition of NO production was required for the iNOS expression and NF-κB translocation. In contrast, resveratrol in a concentration of 30 μmol/l was found to reduce the amount of cytosolic iNOS protein and steady state mRNA levels (Tsai *et al.* 1999), probably due to

inhibition of phosphorylation as well as degradation of IκBα (inhibitory protein bound to NF-κB in its unstimulated form in the cytosol), and a reduced nuclear content of NF-κB subunits. Thus, resveratrol (and other polyphenolic compounds) may inhibit the enhanced expression of iNOS in inflammation through down-regulation of NF-κB binding activity. Bi *et al.* (2005) reported that despite the inhibition of LPS-induced degradation of IκBα, resveratrol may inhibit iNOS expression also *via* suppression of p38 MAPK in microglial cells. The speculation that RES may act more by scavenging of NO radicals and suppressing the generation of RNS than by the inhibition of iNOS gene expression (Chan *et al.* 2000, Manna *et al.* 2000) was not proved in our experiments since RES exhibited only minor NO-scavenging activity in the highest used concentration. Despite that, resveratrol was previously referred to inhibit neutrophil generation of oxidants like superoxide anion and hypochlorous acid (Cavallaro *et al.* 2003).

In conclusion, this study provides the evidence for *in vitro* antioxidative effects of wine polyphenols (CAT, EPI, QUE and RES). It seems that the higher number of hydroxyl substituents is an important structural feature of flavonoids (QUE, CAT, EPI) when compared to hydroxystilbenes (RES) in respect to their direct scavenging activity against ROS and RNS while C-2,3 double bond (present in QUE and RES) might be important for inhibition of ROS and NO production. Only QUE significantly decreased the ROS and NO production in LPS-stimulated RAW 264.7 cells in a dose-dependent manner due to its unique chemical structure.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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