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Flavonoids Protect Against Intercellular Adhesion Molecule-1 Induction by Benzo[a]pyrene

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Abstract Dietary changes are an attractive means of protecting against environmental chemical exposure. Exposure to benzo[a]pyrene (B[a]P) is a risk factor for cardiovascular disease events. It has recently been shown that B[a]P can increase intercellular adhesion molecule-1 (ICAM-1) in endothelial cells, a possible means of promoting cardiovascular disease. This study investigated the ability of flavonoids to protect against B[a]P-induced ICAM-1. It was shown that only flavonoids that contain a 4′ B-ring hydroxyl substitution and a 2–3 C-ring double bond were protective. These data suggest that selected bioactive compounds can decrease proinflammatory properties of environmental chemicals such as B[a]P.

Keywords Benzo[a]pyrene · Flavonoids · Intercellular adhesion molecule-1 · Atherosclerosis

Air pollution is a complex mixture of particles, gases, and organics. The organic fraction is comprised in part by polycyclic aromatic hydrocarbons (PAHs) in which B[a]P is a member. Benzo[a]pyrene B[a]P is a probable human carcinogen that has been linked to the progression of cardiovascular diseases, such as atherosclerosis in both humans and animal models (Burstyn et al. 2005; Curfs et al. 2005). We have shown that B[a]P increases ICAM-1 expression in human endothelial cells through an aryl hydrocarbon receptor (AhR) and caveolae dependent mechanism (Oesterling et al. 2008). ICAM-1 is an immunoglobulin type protein that plays a crucial role in the initiation of atherosclerosis by binding circulating immune cells and causing their migration into the intimal layer of the vessel, culminating in inflammatory foam cell formation (Nageh et al. 1997; Patel et al. 1998).

Treatment of environmental-contaminant induced toxicity is a complicated process. Diet has been suggested as a feasible and economical alternative as a preventative measure (Hennig et al. 2005). One such bioactive compound found extensively in fruits and vegetables is a class of polyphenolic compounds, namely flavonoids (Manach et al. 2004). Flavonoids are divided into groups of compounds, among which are flavones, flavanones, and flavonols. Flavonoids are extensively studied for their antioxidant and anti-inflammatory abilities. However, various studies have shown that flavonoids can be cardioprotective as antioxidants, but also via antioxidant-independent mechanisms (Zhang et al. 2003; Waddington et al. 2004). It was hypothesized that flavonoids would protect vascular endothelial cells against B[a]P-induced ICAM-1 induction.

Methods and Materials

Anti-ICAM-1antibody (clone RR1/1) and secondary AlexaFluor 488 conjugated anti-mouse were purchased from Invitrogen (Carlsbad, CA). B[a]P, beta-naphthoflavone (β-

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NF), kaempferol, apigenin, chrysin, naringenin, hesperetin, galangin, quercetin, luteolin, and propidium iodide were purchased from Sigma–Aldrich (St Louis, MO).

Primary human umbilical vein endothelial cells (HUVEC) were used in these experiments as model for vascular inflammatory diseases. Cells were isolated from human umbilical cord veins as explained previously (Toborek et al. 2002). Human umbilical cords were obtained from the University of Kentucky Labor and Delivery unit and utilized until passage 5. HUVEC were cultured in M199 media (GIBCO Laboratories, Grant Island, NY) supplemented with FBS (Hyclone Laboratories, Logan, UT) as described previously (Toborek et al. 2002).

HUVEC were pre-treated with β-NF (1 μM) and either DMSO or the flavonoid (5 μM) for 16 h. After pretreatment, the cells were treated with DMSO or B[a]P (10 μM) for 24 h. HUVEC were washed with PBS, and then removed with trypsin. Cells were centrifuged, washed, and then incubated in 3% bovine serum albumin containing the primary antibody for ICAM-1 (2 μg/ml) for 30 min. Cells were then centrifuged, washed, and then resuspended in AlexaFluor 488 labeled secondary antibody (3 μg/ml) for 20 min. HUVEC were then washed and stained with propidium iodide (2 μg/ml) for 5 min in order to gate for live cells. Cells were then analyzed by the University of Kentucky Flow Cytometry Facility using a Becton–Dickinson FacsCalibur cell analyzer.

Flavonoids were analyzed for their antioxidant capacity by the measurement of their ferric reducing ability as explained previously (Benzie and Strain 1996; Lotito and Frei 2006). Briefly, flavonoids dissolved in 10% FBS media were combined with a working reagent (1.66 mM $FeCl_3$, 0.83 mM tripyridyltriazine, 300 mM acetate buffer pH 3.6) and the change of absorbance at 593 nm was measured by micro-plate reader (Molecular Devices, SpectroMax M2).

Values are reported as means ± SE of at least three independent groups. Comparisons between treatments were made by one-way analysis of variance followed by Tukey multiple comparison tests using GraphPad Prism 4.0 software (GraphPad Software, San Diego, CA). Statistical probability of $p < 0.05$ was considered significant.

Results and Discussion

A number of epidemiological studies have shown that plant-rich diets are protective against chronic diseases, such as cardiovascular disease (Manach et al. 2005; Dauchet et al. 2006). It is also true that air pollution and the PAHs, such as B[a]P, that are contained within, lead to increases in cardiovascular disease events (Pope et al. 2004; Burstyn et al. 2005). This study reveals that select

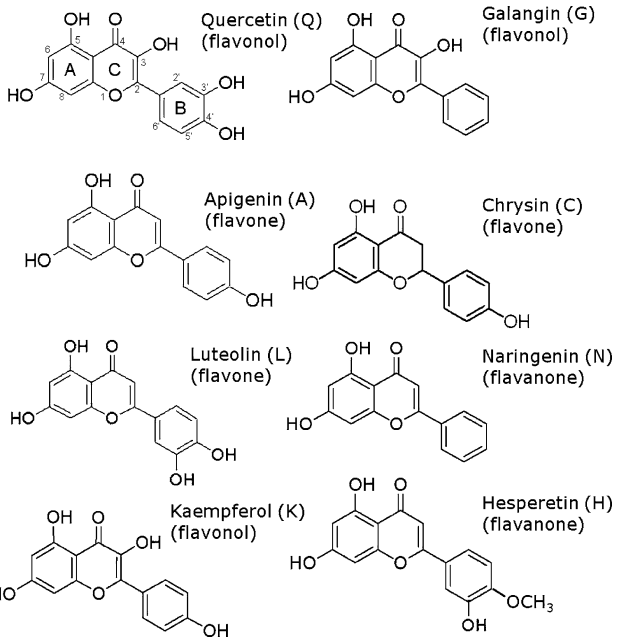

Fig. 1 Structure of flavonoids used in these studies

flavonoids can protect against B[a]P-induced ICAM-1 induction (Fig. 2), which has been shown to be a critical step in the development of cardiovascular diseases. Interestingly, two functional groups appear to be necessary to elicit the protective nature of the compounds, a 2–3 C-ring double bond and a 4′ B-ring hydroxyl substitution. Flavonoids lacking either one of these functional groups were not protective, evidenced by hesperetin, chrysin, naringenin, and galangin (Figs. 1, 2). Past studies have been conducted on the protective role of flavonoids against cytokine induced adhesion molecule expression (Gerritsen et al. 1995; Lotito and Frei 2006). Similarly, the 2–3 C-ring unsaturation was shown to be necessary and similarly apigenin was found to be a potent protector (Gerritsen et al. 1995; Lotito and Frei 2006). However, most of these studies used high, physiologically irrelevant concentrations of flavonoids (up to 50 μM) and none of them have investigated other inducers of adhesion molecules such as environmental contamination.

One of the key characteristics of flavonoids that is heavily studied is the antioxidant ability of these compounds. Some studies have suggested that flavonoids elicit their protective effects through their antioxidant capability (Kris-Etherton et al. 2004; Dauchet et al. 2006) while others have suggested that the protection is independent of their antioxidant roles (Zhang et al. 2003; Waddington et al. 2004). ICAM-1 can be up-regulated by the activation of redox sensitive transcription factors, thus antioxidants have been suggested as protective against adhesion molecule production. We examined the ability of the eight flavonoids tested to reduce ferric iron and then compared

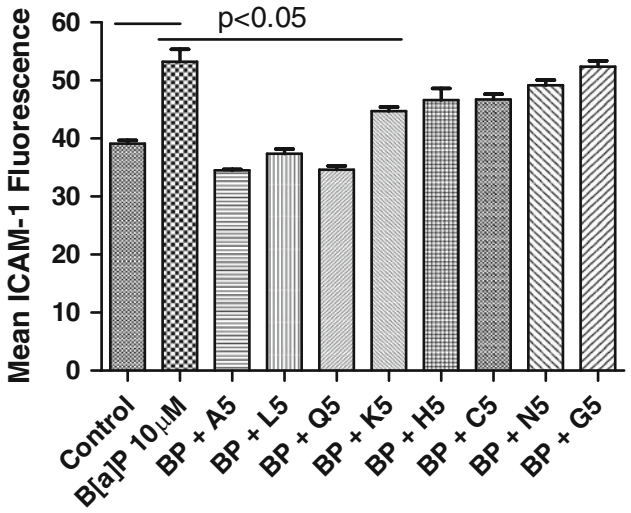

Fig. 2 Select flavonoids protect against B[a]P-induced ICAM-1. HUVEC were pre-treated with 1 μM β-NF and 5 μM flavonoids (apigenin (A), luteolin (L), quercetin (Q), kaempferol (K), hesperetin (H), chrysin (C), naringenin (N), galangin (G)), followed by a 24 hour treatment with DMSO or B[a]P (10 μM). Cells were labeled with mouse anti-human ICAM-1 and AlexaFluor 488 antibodies. Positively labeled cells were analyzed by the flow cytometry. Bars represent mean ± SE of at least three independent experiments analyzed by one-way ANOVA followed by Tukey multiple comparison test

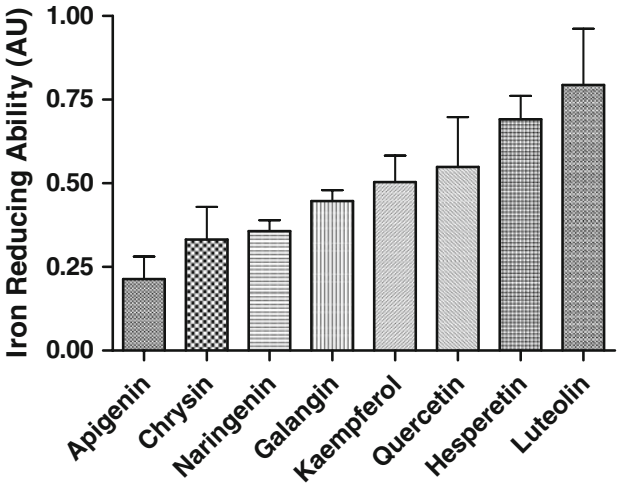

Fig. 3 Antioxidant ability of flavonoids. Flavonoids (5 μM) were analyzed for their antioxidant capacity by the measurement of their ferric reducing ability. Results are expressed as mean ± SE of absorbance units (AU) of at least three independent experiments

the reducing capacity to their ability to protect against B[a]P-induced ICAM-1 (Fig. 3). Apigenin was the least capable of reducing iron whereas luteolin was the strongest antioxidant tested. The antioxidant ability of each flavonoid did not correspond to it's ability to protect against B[a]P. For example, apigenin was the most protective flavonoid against B[a]P-induced ICAM-1, however apigenin had the least antioxidative properties. The reverse is true for hesperetin which showed a strong antioxidant ability but did not protect against B[a]P. It appears as though the flavonoids with numerous B-ring substitutions were more likely to be strong antioxidants (Fig. 1). It has been suggested that the hydroxyl substitution on the C-ring is an important functional group for antioxidant capacity, however this was not true for this study (Lotito and Frei 2006) evidenced by the lack of this substitution on both luteolin and hesperetin.

One possibility for the protective nature of the flavonoids is the antagonism of the aryl hydrocarbon receptor (AhR). Studies have suggested that the B-ring 4′ substitution of the flavonoids is important for interaction with the AhR ligand binding site and that it could create a hydrogen bond between the compound and the AhR amino acids (Gasiewicz et al. 1996; Zhang et al. 2003; Puppala et al. 2007). B[a]P is extensively metabolized by AhR mediated xenobiotic metabolizing enzymes such as cytochrome P450 1A1 and epoxide hydrolase. It has been shown by our lab that B[a]P must be metabolized by AhR controlled enzymes to induce ICAM-1 (Oesterling et al. 2008). It is possible that the flavonoids, acting as AhR antagonists, are able to circumvent the metabolism of B[a]P to the metabolically active compounds, thus protecting the cells from these events. This study also showed the necessity of the 2–3 double bond of the flavonoids for protection. This functional group was also shown to be important in cytokine induced ICAM-1 studies suggesting that the double bond is more important to ICAM-1 induction and less to the specific inducer, whereas the 4′ hydroxyl substitution is unique to AhR metabolized compounds such as B[a]P.

In summary, our data provide evidence that plant-derived flavonoids can down-regulate proinflammatory parameters induced by B[a]P. Furthermore, our data suggest that the protective properties of selected flavonoids initiate at the level of AhR, possibly by competing as ligands with B[a]P. Nutrition may be a sensible means of reducing health risks associated with exposure to environmental pollutants such as B[a]P.

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