Cardiovascular disease and nutritional phenolics

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1 Introduction

Arteriosclerosis is a chronic pathogenic inflammatory-fibro-proliferative process of large and medium-sized arteries that results in the progressive formation of fibrous plaques, which in turn impair the blood flow of the vessel. These lesions can either promote an occlusive thrombosis in the affected artery or produce a gradual but relentless stenosis of the arterial lumen. In the first case, an infarction of the organ supplied by the afflicted vessel occurs, such as in a heart attack, when a coronary artery is affected, and in a thrombotic stroke when a cerebral artery is suddenly blocked. In the second case, the stenosis of the vessel leads to a progressive and gradual damage of the affected organ part.

A number of subtle dysfunctions occur at the cellular and molecular levels in the early stages of disease progression associated with the loss of cellular homeostatic functions of endothelial cells, smooth muscle cells and macrophages which constitute the major cell types in the atheroma environment. These events include the modification of the pattern of gene expression, cell proliferation and apoptosis.

In the last few decades, several epidemiological studies have shown that a dietary intake of foods rich in natural antioxidants correlates with reduced risk of coronary heart disease; particularly, a negative association between consumption of polyphenol-rich foods and cardiovascular diseases has been demonstrated. This association has been partially explained on the basis of the fact that polyphenols interrupt lipid peroxidation induced by reactive oxygen species (ROS). A large body of studies has shown that oxidative modification of the low-density fraction of lipoprotein (LDL) is
implicated in the initiation of arteriosclerosis. More recently, alternative mechanisms have been proposed for the activity of antioxidants in cardiovascular disease, which are different from the ‘simple’ shielding of LDL from ROS-induced damage. Several polyphenols recognised for their antioxidant properties might significantly affect cellular response to different stimuli, including cytokines and growth factors.

2 LDL oxidation and atherogenesis

At cellular level each stage of atheroma development is accompanied by the expression of specific glycoproteins by endothelial cells which mediate the adhesion of monocytes and T-lymphocytes. Their recruitment and migration is triggered by various cytokines released by leukocytes and possibly by smooth muscle cells. Atheroma development continues with the activation of macrophages, which accumulate lipids and become, together with lymphocytes, so-called fatty streaks. The continuous influx, differentiation and proliferation finally leads to more advanced lesion and to the formation of the fibrous plaque.

It is accepted that oxidation of LDL is a key event in endothelial injury and dysfunction. Oxidised LDL (oxLDL) may directly injure the endothelium and trigger the expression of migration and adhesion molecules. Monocytes and lymphocytes interact with oxLDL and the phagocytosis which follows leads to the formation of foam cells, which in turn are associated with the alteration of the expression pattern of growth regulatory molecules, cytokines and pro-inflammatory signals. The proposed role of oxLDL in atherogenesis, based on studies in vitro, is shown in Fig. 5.1.

LDL, modified by oxidation, glycation and aggregation, is considered a major cause of injury to the endothelium and underlying smooth muscle. LDL, entrapped in the subendothelial space, can undergo progressive oxidation (minimally modified-LDL, mm-LDL). Once modified, LDL activates the expression of molecules entitled for the recruitment of monocytes and for the stimulation of the formation of monocyte colonies (monocyte chemotactic protein, MCP-1; monocyte colony stimulating factor M-CSF) in the endothelium. These molecules promote the entry and maturation of monocytes to macrophages, which further oxidise LDL. Modified LDL is also able to induce endothelial dysfunction, which is associated with changes of the adheresiveness to leukocytes or platelets and the wall permeability. Dysfunctional endothelium also displays pro-coagulant properties and the expression of a variety of vaso-active molecules, cytokines, and growth factors. LDL, oxidised in vitro by several cell systems or by cell-free systems (transition metal ions or azo-initiators), is recognised by the scavenger receptor of macrophages. The increasing affinity of LDL for the scavenger receptor is associated with changes in its structural and biochemical properties, such as the formation of lipid hydroperoxides, oxida-
5.1 Sequence of events in atherogenesis and role of low-density lipoprotein. Native LDL, in the subendothelial space, undergoes progressive oxidation (mmLDL) and activates the expression of MCP-1 and M-CSF in the endothelium (EC). MCP-1 and M-CSF promote the entry and maturation of monocytes to macrophages, which further oxidise LDL (oxLDL). Ox-LDL is specifically recognised by the scavenger receptor of macrophages and, once internalised, formation of foam cells occurs. Both mmLDL and oxLDL induce endothelial dysfunction, associated with changes of the adhesiveness to leukocytes or platelets and to wall permeability.

tive modification and fragmentation of apoprotein B-100 and an increase of negative charge. The exact mechanism of LDL oxidation in vivo is still unknown, but transition metal ions, myeloperoxidase, lipoxygenase, and nitric oxide are thought to be involved.

3 Polyphenols and cell response

Plants produce a variety of secondary products containing a phenol group, i.e. a hydroxyl group on an aromatic ring. These compounds are of a chemically heterogeneous group that includes simple phenols, flavonoids, lignin and condensed tannins. About four thousand plant substances belong to the flavonoid class, of which about 900 are present in the human diet. The daily intake of flavonoids in Western countries has been estimated to be about 23mg per day. No analogous calculation has been done for phenolic acids but it is likely to be quite similar in the western diet.

Many studies have been undertaken to establish the structural criteria for the activity of polyhydroxy flavonoids in enhancing the stability of fatty
acid dispersions, lipids, oils, and LDL.\textsuperscript{20,21} As for phenolic acids, the inhibition of oxidation by flavonoids is related to the chelation of metal ions via the ortho-dihydroxy phenolic structure, the scavenging of alkoxy and peroxy radicals, and the regeneration of \( \alpha \)-tocopherol through reduction of the tocopheryl radical.\textsuperscript{20} The contribution of flavonoids and phenolic acids to the prevention and possibly to the therapy of cardiovascular disease can also be found on metabolic pathways other than the antioxidant capacity. As previously mentioned, arteriosclerosis is characterised by early cellular events and by the dysregulation of the normal cellular homeostasis.\textsuperscript{17} Molecular mechanisms, by which polyphenols may play a role either in the etiopathology or in the pathophysiology of arteriosclerosis, will be discussed here, with particular regard to the modulation of gene expression regulated by the transcription factor nuclear factor-kappa B (NF-\( \kappa \)B), and to the induction of either apoptotic or proliferative responses.

4 Polyphenols and activated NF-\( \kappa \)B

The transcription factors of the nuclear factor-\( \kappa \)B/Rel family control the expression of a spectrum of different genes involved in inflammatory and proliferation responses. The typical NF-\( \kappa \)B dimer is composed of the sub-units p50 and p65, and it is present as its inactive form in the cytosol bound to the inhibitory proteins I\( \kappa \)B. Following activation by various stimuli, including inflammatory or hyperproliferative cytokines, ROS, oxidised LDL and bacterial wall components, the phosphorylation and proteolytic removal of I\( \kappa \)B from the complex occurs. The activated NF-\( \kappa \)B immediately enters the nucleus where it interacts with regulatory \( \kappa \)B elements in the promoter and enhancer regions, thereby controlling the transcription of inducible genes.\textsuperscript{22,23} A spectrum of different genes expressed in arteriosclerosis have been shown to be regulated by NF-\( \kappa \)B, including those encoding TNF-\( \alpha \), IL-1, the macrophage or granulocyte colony stimulating factor (M/G-CSF), MCP-1, c-myc and the adhesion molecules VCAM-1 and ICAM-1.\textsuperscript{24} In the early stages of an atherosclerotic lesion, different types of cells (macrophages, smooth muscle cells and endothelial cells) interplay to cause a shift from the normal homeostasis and a vicious circle may be triggered, exacerbating dysfunction. Figure 5.2 shows a sketch of the regulation of NF-\( \kappa \)B activation by oxidants/antioxidants. Some of major genes involved in the atherogenesis are also listed.

Several lines of evidence, including the inhibition by various antioxidants, suggest that NF-\( \kappa \)B is subject to redox regulation. Because of its pivotal role in inflammatory response, a significant effort has focused on developing therapeutic agents that regulate NF-\( \kappa \)B activity. In this scenario polyphenols may play an important role, either by directly affecting key steps in the activation pathway of NF-\( \kappa \)B, or by modulating the intracellular redox status, which is, in turn, one of the major determinants of NF-\( \kappa \)B
Consistently, experimental data are accumulating regarding polyphenolic compounds as natural phytochemical antioxidants that possess anti-inflammatory properties by downregulating NF-κB. Some of the most relevant findings about this aspect are summarised in Table 5.1.

5 Other aspects of polyphenols as modulators of signal transduction

Several studies have demonstrated that depending on their structure, flavonoids may be inhibitors of several kinases involved in signal transduction, mainly protein kinase C (PKC) and tyrosine kinases.26–29 Agullo et al.30 tested 14 flavonoids of different chemical classes and reported that myricetin, luteolin and apigenin were efficient inhibitors of phosphatidylinositol 3-kinase, PKC and tyrosine kinase activity. The authors also observed a structure–function in that the position, number and substitution of hydroxyl groups on the B ring and the saturation of C2–C3 bonds affect activation.6,25
Table 5.1 Flavonoids and flavonoid-related compounds suppressing NF-κB activity in cell culture studies

<table>
<thead>
<tr>
<th>Name</th>
<th>Concentration (duration)</th>
<th>Inducers</th>
<th>Cell lines</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apigenin</td>
<td>25 μM (4h)</td>
<td>TNFα, TNFα + IFNγ</td>
<td>HUVEC</td>
<td>[Gerritsen et al., 1995]</td>
</tr>
<tr>
<td>Caffeic acid phenethyl ester (propolis)</td>
<td>25 μg/ml (2h)</td>
<td>TNFα, PMA, Ceramide-C8, Okadaic acid</td>
<td>U937</td>
<td>[Natarajan et al., 1996]</td>
</tr>
<tr>
<td>Epigallocatechin-3-gallate (green tea)</td>
<td>15 μM (Co-incubation with inducer)</td>
<td>LPS</td>
<td>Mouse peritoneal macrophages</td>
<td>[Lin et al., 1997]</td>
</tr>
<tr>
<td></td>
<td>100 μM (2h)</td>
<td>LPS</td>
<td>RAW 264.7</td>
<td>[Yang et al., 1998]</td>
</tr>
<tr>
<td>Genistein (soy, clover)</td>
<td>148 μM (1–2h)</td>
<td>TNFα, Okadaic acid</td>
<td>U937, Jurkat, HeLa</td>
<td>[Natarajan et al., 1998]</td>
</tr>
<tr>
<td>Ginkgo biloba extract</td>
<td>100–400 μg/ml (18h)</td>
<td>H2O2</td>
<td>PAEC</td>
<td>[Wey et al., 1999]</td>
</tr>
<tr>
<td>Quercetin (wine, onion)</td>
<td>265 μM (1h)</td>
<td>TNFα</td>
<td>U937</td>
<td>[Natarajan et al., 1998]</td>
</tr>
<tr>
<td></td>
<td>10 μM (co-incubation with inducer)</td>
<td>H2O2</td>
<td>HepG2</td>
<td>[Musonda and Chipman, 1981]</td>
</tr>
<tr>
<td>Silymarin (Silybum marianum)</td>
<td>12.5 μg/ml (24h)</td>
<td>Ultraviolet, Okadaic acid, LPS, TNFα, TNFα</td>
<td>HaCaT, HepG2, Würzburg, U937, HeLa, Jurkat</td>
<td>[Saliou et al., 1999], [Saliou et al., 1998], [Manna et al., 1999]</td>
</tr>
<tr>
<td>Taxifolin (pine bark)</td>
<td>303 μM (24h)</td>
<td>IFNγ</td>
<td>RAW 264.7</td>
<td>Park et al., 2000</td>
</tr>
<tr>
<td>Theaflavin-3,3’-digallate (black tea)</td>
<td>10 μM (co-incubation with inducer for 1h)</td>
<td>LPS</td>
<td>RAW 264.7</td>
<td>[Lin et al., 1999]</td>
</tr>
</tbody>
</table>
flavonoid activity on different kinases. Wolle et al.\textsuperscript{31} examined the effect of flavonoids on endothelial cell expression of adhesion molecules. A synthetic flavonoid, 2-(3-amino-phenyl)-8-methoxy-chromene-4-one, an analog of apigenin, markedly inhibited TNF-\(\alpha\)-induced VCAM-1 cell surface expression in a concentration-dependent fashion, but had no effect on ICAM-1 expression. The inhibition correlated with decreases in steady state mRNA levels, resulting in a reduction in the rate of gene transcription rather than changes in mRNA stability. No effects on NF-\(\kappa\)B activation were observed either by mobility shift assay or by reporter gene assay, indicating that the modulation of VCAM-1 gene expression is due to a NF-\(\kappa\)B-independent mechanism. More recently, Nardini et al. reported that both caffeic acid and the procyanidin-rich extract from the bark of \textit{Pinus maritima} inhibit \textit{in vitro} the activity of phosphorylase kinase, protein kinase A and protein kinase C.\textsuperscript{32} Taken together, these studies opened an important issue in the ability of polyphenols to modulate the expression of genes responsible for pro-atherogenic processes with or without altering the activity of NF-\(\kappa\)B, which can be considered fundamental for other cellular functions.

Hu et al.\textsuperscript{33} reported that oncogene expression (c-myc, c-raf and c-H-ras) \textit{in vivo}, induced by nitrosamine treatment, is inhibited in mouse lung by tea drinking. The same authors also reported that topical pre-treatment with the tea flavonoid (\(-\))-epigallocatechin gallate significantly inhibits oncogene expression induced by PMA in mouse skin.\textsuperscript{33} Similarly, c-fos expression, cell growth and PKC activity induced by PMA in NIH3T3 cells were inhibited by the natural flavonoid apigenin, as reported by Huang et al.\textsuperscript{34} Green tea polyphenol extract stimulates the expression of detoxifying enzymes through antioxidant responsive element in the cultured human hepatoma cell line HepG2.\textsuperscript{35} This activity seems to be mediated by potentiation of the mitogen activated protein kinases (MAPKs) signalling pathway, suggesting an indirect activity of polyphenols in the regulation of cellular responses to oxidative injury. Lin et al.\textsuperscript{36} reported that both curcumin and apigenin inhibit PKC activity induced by PMA treatment in mouse skin. The same inhibitory effect can be observed in mouse isolated fibroblasts pretreated with curcumin. Apigenin, kaempferol and genistein reverted the transformation of the \(v\)-H-ras transformed NIH3T3 line. The authors suggest that both PKC activity and oncogene expression may be the mechanism by which polyphenols exert their anti-tumor activity.\textsuperscript{36} The flavonoid silymarin inhibits the expression of TNF-\(\alpha\) mRNA induced by either 7,12-dimethylbenz(a)anthracene or okadaic acid in the SENCAR mouse skin model.\textsuperscript{37} This inhibitory activity, which is associated with a complete protection of mouse epidermis from tumour promotion by OA and results in a significant reduction (up to 85\%) of tumour incidence induced by 7,12-dimethylbenz(a)anthracene,\textsuperscript{26} may also be relevant in the atherogenesis, since TNF-\(\alpha\) plays a central role in the vicious circle of macrophage-endothelial cell dysfunction.\textsuperscript{24,38}
The cell-to-cell interaction following the expression of adhesion molecules (ICAM-1, VCAM-1 and selectin) in endothelial cells induced by cytokines treatment has been reported to be blocked by hydroflavones and flavanols. Apigenin, the most potent flavone tested in this study, inhibited the expression of adhesion molecules, the expression of both interleukin-6 and interleukin-8 induced by TNF-α and interleukin-1-induced prostaglandin synthesis. Apigenin was found to have no effect on the nuclear translocation of NF-κB, but significantly inhibited the expression of the reporter gene β-galactosidase driven by NF-κB elements in SW480 cells induced by TNF-α, suggesting that NF-κB transcriptional activation was affected. Also the adhesion of cytokine treated lymphocytes to endothelial cells was blocked by pretreatment of endothelial cells with apigenin. Finally, the same study reports apigenin to have a strong anti-inflammatory activity in vivo on carragenin-induced rat paw edema and on delayed type hypersensitivity in the mouse. Taken together, these data suggest that both flavonoids and phenolic acids may have important effects in diseases involving leukocyte adhesion and trafficking and oxidant-induced gene expression.

6 Indirect evidence for polyphenol activity in atherogenesis

An indirect effect of flavonoids and phenolic acids on NF-κB activation, and therefore on NF-κB-driven gene expression, may be inferred from two kinds of study: one addressing the modulation of NF-κB activity by other antioxidant molecules (α-tocopherol, thiolic antioxidants such as N-acetyl-cysteine, lipoic acid, pyrrolidendithiocarbamate), and others addressing the role of flavonoids and phenolic acids in the antioxidant network. α-Tocopherol and lipoic acid inhibit NF-κB in different cellular models, and several studies describe the ability of flavonoids and phenolic acids to exert a significant tocopherol and glutathione sparing effect either under basal homeostatic conditions or following oxidative challenge.

Roy and co-workers demonstrated that the adhesion of lymphocyte to endothelial cells is regulated by the thiolic antioxidant α-lipoic acid and by α-tocopherol. Similarly, an enhancement of the endogenous levels and a protective effect on α-tocopherol after peroxynitrite treatment by the procyanidin-containing extract from pine bark was reported by Virgili et al. The same complex mixture of procyanidins has been reported to enhance the activity of the enzymatic machinery which regulates the GSH redox status in endothelial cells. In fact, a significant increase in GSH (reduced glutathione) levels, an increased activity of the GSH redox enzymes (GSH reductase and GSH peroxidases) and an increase in the enzymatic activity of both SOD (superoxide dismutase) and catalase have been reported and proposed by Wei and collaborators to be mediated by
an increase of protein synthesis. The important role of GSH in the antioxidant network usually results in a greater resistance to pro-oxidant cytotoxicity and, in general, leads to a greater resistance of cells to dysfunction.

Proliferation of vascular smooth muscle cells is one of the most important features of arteriosclerosis. Vascular smooth muscle cells display a unique susceptibility to antioxidants which indicates that they respond differently from other types to changes in the redox status. In fact, hydrogen peroxide has been demonstrated to stimulate the proliferation of vascular smooth muscle cells while inhibiting the proliferation of vascular endothelial cells. However, the effect of antioxidants on smooth muscle cell proliferation is still unclear. α-Tocopherol inhibits the proliferation of smooth muscle cells by preventing the activation of PKC. Two structurally different thiol-containing compounds, N-acetylcysteine (NAC) and pyrrolidinedithiocarbamate (PDTC) have been reported to induce apoptosis in cultured vascular smooth muscle cells in a dose- and time-dependent fashion. In the same report the overexpression of the proto-oncogene bcl-2 was observed to counter PDTC and NAC-induced apoptosis, suggesting that thiol oxidation status in the cell plays an important role in switching on the apoptotic program.