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Polyphenolic Flavonoids and Atherosclerosis: Cellular Mechanisms in Early Events of Atherogenesis

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INTRODUCTION

Much attention has been directed towards human behavioural habits that could be considered risk factors for developing chronic pathologies. In particular, much effort has been devoted to elucidate the role of diet in preventing cardiovascular diseases. A so-called "Mediterranean diet" is thought to prevent cardiovascular diseases as a consequence of its high content of antioxidants, which are crucial in ameliorating oxidative events implicated in many diseases. Similarly, moderate consumption of red wine has been associated with a lowering of the risk of coronary heart disease (French Paradox).

There is currently intense interest in polyphenolic phytochemicals such as flavonoids, proanthocyanidins and phenolic acids. Epidemiological studies have evidenced that an increased intake of polyphenolic phytochemicals such as flavonoids, proanthocyanidins and phenolic acids found in a large number of fruits and vegetables, may contribute to the low incidence of cardiovascular diseases (1,2), and this phenomenon appears to be associated with their antioxidant capacity (3). There are several subclasses such as flavonols, flavones, isoflavones, flavonones, flavan-3-ols, and anthocyanidins. These flavonoids are natural antioxidants that scavenge various types of radicals in aqueous and organic environments (4-6). Whether these flavonoids act *in vivo* as antioxidants or anti-inflammatory agents appears to depend on their bioavailabilities.

It has been shown that there are differences in the antioxidant capacity among different groups of flavonoids, and within each group of flavonoids (5,7). Because flavonoids exert cardioprotective effects that are not only due to antioxidant/anti-radical/anti-inflammatory activity, the present data described here deal with the effects of flavonoids at vascular single cell level (human endothelial cells) with special emphasis on the anti-apoptotic and the anti-atherogenic properties. In particular, four different subclasses of polyphenolic flavonoids are compared in the vascular endothelium model: flavanols [(-)epigallocatechin gallate and (+)catechin]; flavonols (quercetin and myricetin); flavanones (naringin and hesperidin); and flavones (luteolin and apigenin).

Anti-Apoptotic Actions of Flavonoids

Oxidative injury following diverse stimuli including clinical and experimental ischemia/hypoxia, reperfusion, and inflammation can induce cardiac and endothelial cell apoptosis (8,9), which is involved in the atherogenic lesion formation. Apoptosis is indispensable for physiological development and homeostasis of tissues and elimination of diseased cells in multi-organisms (10,11). Defects in apoptosis have been also implicated in neurodegenerative diseases, cancer, and autoimmune diseases (11,12). Accordingly, agents or antioxidants that can inhibit production of reactive oxygen species can prevent apoptosis (8,13). However, the underlying molecular mechanisms by which anti-oxidative agents protect cells from stimulator-triggered apoptosis remain to be elucidated.

Different groups of flavonoids exhibit a scavenging activity against DPPH radical with different SC_{50} in the

cell-free system; flavanols and flavonols are the most potent flavonoids, and flavanones are inactive in scavenging the radical. Some groups of flavonoids, e.g., flavanols and flavonols, exhibit a more powerful spontaneous antioxidant capacity scavenging the DPPH radical than the classic antioxidant L-ascorbic acid in the cell-free systems, while other flavonoids, especially apigenin, are not effective. It is assumed that there is a relationship between their structure and DPPH scavenging activity (5,6).

The flavanol (-)epigallocatechin gallate and the flavonol quercetin at the non-toxic dose of 50 mol/L prevent H_2O_2-induced injury and prolonged endothelial cell survival. It has been shown that the flavones of luteolin and apigenin intensify H_2O_2-induced endothelial apoptosis. These findings are consistent with previous reports showing apoptotic death processes in various types of cells induced by H_2O_2 (14). Agents that inhibit production of reactive oxygen species or enhance cellular antioxidant defenses can prevent apoptosis and protect cells from the damaging effects of oxygen radicals (8,13). Consistent with these reports, the flavanols and flavonols, in particular (-)epigallocatechin gallate and quercetin, have high anti-apoptotic activities in the H_2O_2-treated vascular endothelial cells. In contrast, at non-toxic doses the flavone-type flavonoids have no anti-apoptotic effects and intensify the apoptosis-like alterations including nuclear condensation and DNA fragmentation. These results are in agreement with those obtained for DPPH scavenging activity, indicating that there is a major structural feature responsible for the anti-apoptotic activity against reactive radicals. In addition, the anti-apoptotic activities of polyphenolic flavonoids prove to be diverse.

Rodent feeding studies have supported the possibility that certain flavonoids may have antioxidant functions *in vivo* (15,16). However, the underlying mechanisms for their cardio- and cytoprotective actions are still unknown. Their antioxidant actions in the oxidant-induced endothelial apoptosis have been mediated through their H^+-donating properties (17). However, not all flavonoids exhibit anti-apoptotic activity in the H_2O_2-treated cells, suggesting that other mechanisms for cytoprotection against oxidant insults must be involved.

(-)Epigallocatechin gallate and quercetin restore expression of the anti-apoptotic Bcl-2 protein and increase expression of the pro-apoptotic Bax protein in response to H_2O_2, providing compelling evidence in support of their potent anti-apoptotic actions. It has been proposed that reactive oxygen species down-regulates the anti-apoptotic Bcl-2 and up-regulates the pro-apoptotic Bax. Bcl-2 expressing cells have been reported to have the enhanced antioxidant capacity that suppresses oxidative stress signals (18,19).

H_2O_2-induced caspase-3 cleavage in endothelial cells is blocked by (-)epigallocatechin gallate and quercetin. The substantial difference between these flavonoids in inhibiting the activated caspase-3 appears to be responsible for the difference in their anti-apoptotic activities. (-)Epigallocatechin gallate and quercetin appear to switch off the apoptotic death cascade by inhibiting the activation of caspase-3 and likely by enhancing the intrinsic cellular tolerance against apoptotic triggers. Conversely, the H_2O_2-activated caspase-3 is sustained in apigenin-treated cells showing no anti-apoptotic efficacy. Thus, our findings suggest that the anti-apoptotic flavonoids may function by acting selectively through various endothelial death signaling cascades. In addition, flavonoids affect multiple signaling pathways that converge at the level of transcriptional regulation, i. e. MAPK-responsive pathways (20).

Early Anti-Atherogenic Actions of Flavonoids

Dietary intakes of flavonols, flavones and isoflavones by Japanese women are inversely correlated with the plasma LDL cholesterol concentration (21). Flavonoids and related polyphenolics have a great potential to delay LDL oxidation with the radical scavenging capacity (22). Wine flavonoids have been shown to protect against atherosclerosis by inhibiting the accumulation of oxidized LDL in atherosclerotic lesions, paraoxonase elevation

and removal of atherogenic lesions of apolipoprotein E deficient mice (23). This observation implies that flavonoids confer protection against early events in atherogenic lesion formation.

It has been documented that flavonoids act as anti-inflammatory agents to inhibit expression of cell adhesion molecules and matrix proteases (24,25). Induction of CAM is a common feature in inflammatory environments and occurs in the early development of atherosclerosis. Cell adhesion molecules such as VCAM-1, ICAM-1 and E-selectin have been observed in atherosclerotic lesions and at sites predisposed to lesion formation in rabbit and mouse as well as in human coronary atherosclerotic plaques (26).

Different subgroups of flavonoids have different efficacies in inhibiting TNF-α-induced monocyte adhesion; the flavones are the most potent flavonoids, and the flavanols and flavanones are ineffective in preventing monocyte adherence on TNF-α-activated endothelial cells. Flavonoids may inhibit early events in the atherosclerotic process by modulating monocyte adhesion and transmigration. Although definite mechanisms underlying the flavonoid protection against early atherogenic process are not fully understood, they may involve down-regulation of inflammatory chemokines and cytokines, matrix proteases and cell adhesion molecules (24,25). The flavanones, naringenin and hesperetin and their glycosides, hesperidin and naringin have no inhibitory activity, implying that the presence of rutinose moiety of flavanones does not facilitate blocking monocyte adhesion on the activated endothelium.

The flavones, luteolin and apigenin at the non-toxic dose of =10 μmol/L, near-completely block expression of VCAM-1, ICAM-1 and E-selectin proteins via a direct modulation at their gene transcriptional levels. The flavonol quercetin substantially attenuates expression of these cell adhesion molecules at =25 μmol/L, while flavanol catechins, and the flavanones of hesperetin and naringenin and their glycosides do not have such inhibitory activity even at 50 μmol/L. The ability of these flavonoids to block TNF-α-induced cell adhesion molecule expression could be due to their antioxidant capacity. It has been demonstrated that oxidative stress up-regulates VCAM-1 and E-selectin expression via redox-sensitive transcriptional activation and is inhibited by the antioxidants pyrrolidine dithiocarbamate and N-acetylcysteine (27,28). Also classical antioxidant vitamin E has been shown to inhibit expression of cell adhesion molecules and adhesion of monocytes to endothelial cells (29,30). (-)Epigallocatechin gallate has been previously shown to have a potent antioxidant activity. Nevertheless, this flavanol did not prevent pro-inflammatory agent-induced monocyte adhesion and cell adhesion molecule expression. Western blot analysis shows that 5 μmol/L N-acetylcysteine did not down-regulate TNF-α-induced VCAM-1 expression. Thus, it is unlikely that the antioxidant activity of flavonoids contributes to their blockade of endothelial cell adhesion molecule induction by TNF-α and to the atheroprotective actions of flavones. Luteolin has been recently reported to reduce lipopolysaccharide-induced lethal toxicity possibly by inhibiting TNF-α and ICAM-1 expression *in vivo* (31).

The flavones ppear to inhibit the cell adhesion molecule expression by blunting the activation of NF-κB stimulated by TNF-α. The inhibitory mechanism(s) of these flavonoids are inferred from the possibility that flavonoids may interrupt a signaling cascade involving cell adhesion molecule transcription activation of NF-κB which plays an important role in the inducible regulation of cellular inflammatory genes and which is activated in atherosclerotic tissues (32). Phenolic gallate has previously been shown to inhibit cytokine-induced nuclear translocation of NF-κB and expression of leukocyte adhesion molecules in endothelial cells (33). In addition, it has been shown that luteolin abolishes lipopolysaccharide-induced increase in inhibitory protein IκB-α phosphorylation, NF-κB-mediated gene expression and pro-inflammatory cytokine production in murine macrophages (34). The anti-inflammatory activity of resveratrol, a naturally occurring phytoalexin found in grapes and wine, could be mediated by its interference with NF-κB-dependent transcription (35). In our study, quercetin, luteolin and apigenin attenuate

or block nuclear translocation of p65 and DNA binding activity of NF-κB stimulated by TNF-α, which in turn attenuate cell adhesion molecule expression at the transcriptional levels. However, the mechanisms underlying this inhibition are still uncertain. Quercetin has been reported to inhibit ICAM-1 expression induced by phorbol 12-myristate 13-acetate or TNF-α without NF-κB activation in human endothelial cell line ECV304 (36). In addition, a flavonoid 2-(3-amino-phenyl)-8-methoxy-chromene-4-one selectively blocks TNF-α-induced VCAM-1 expression in human aortic endothelial cells by NF-κB-independent mechanism (37).

CONCLUSION

The endothelial cell model demonstrates that there are differences in anti-apoptotic capacity among flavonoids, which appears to stem from their disparate chemical structures. Unlike flavones, the flavanol (-)epigallocatechin gallate and the flavonol quercetin protect the endothelium from oxidant-induced apoptosis. This anti-apoptotic protection is possibly mediated, at least in part by a mechanism linked to pro-apoptotic Bax blockade and anti-apoptotic Bcl-2 activation. It is crucial to elucidate the precise sites of action of anti-apoptotic flavonoids in the sequence of vascular injury events that regulate oxidant-induced cell death and to further evaluate the potential of dietary flavonoids as cardio- and cytoprotective agents. Furthermore, the capability of flavonoids to block the early atherogenic process involving endothelial expression of inducible adhesion molecules differs among individual flavonoid subclasses. Quercetin and flavones block monocyte adhesion on the TNF-α-activated endothelium and the activation of cell adhesion molecule expression. The selective inhibition of cell adhesion molecule expression by quercetin and flavones is at least in part mediated via the regulation of translocation and transactivation of NF-κB. This observation might have implications for strategies preventing and attenuating inflammatory diseases. These inhibitory mechanisms of the flavones appear to be independent of an antioxidant sensitive transcriptional regulatory mechanism and may argue for transcriptional mechanisms as the major target of anti-atherogenic action of flavones.

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