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MAPK signaling in neurodegeneration: influences of flavonoids and of nitric oxide

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Abstract

Oxidative and nitrosative stress is increasingly associated with the pathology of neurodegeneration and aging. The molecular mechanisms underlying oxidative/nitrosative stress-induced neuronal damage are emerging and appear to involve a mode of death in which mitogen-activated protein kinase (MAPK) signaling pathways are strongly implicated. Thus, attention is turning towards the modulation of intracellular signaling as a therapeutic approach against neurodegeneration. Both endogenous and dietary agents have been suggested as potent modulators of intracellular signal transduction, e.g. nitric oxide and flavonoids, respectively. This review addresses recent findings on the biological effects of flavonoids and nitric oxide in neurodegeneration and aims to elucidate the rationale for their prospective use as modulators of cellular signal transduction.

Keywords: Apoptosis; Oxidative stress; Neuron; Flavonoids; MAP kinase; JNK; ERK; Epicatechin; Nitric oxide; Mitochondria; Redox status; Polyphenols

1. Oxidative/nitrosative stress

The pathology of neurodegeneration and aging is increasingly associated with oxidative and nitrosative stress mediated by reactive oxygen species (ROS) and reactive nitrogen species (RNS) [64,77], thus implicating the use of antioxidants as potentially beneficial strategies. The redox state of the cell is the net balance between the steady-state levels of ROS and RNS and the cellular antioxidant systems. A key indicator of this balance is the ratio GSH/GSSG, or more accurately, the GSH redox potential. The GSH/GSSG pool is dynamic, undergoing reversible oxidation–reduction reactions with ROS and RNS, antioxidant systems and protein thiols. Furthermore, it appears that cells become more oxidized during the progression of the life cycle from the proliferative state (−230 to 260 mV), through cell cycle arrest and differentiation (−200 mV) to apoptosis (−150 mV). The latter trend is paralleled by a progressive increase in the cellular quasi steady-state level of H_2O_2, with expected changes in the subcellular H_2O_2 gradients across membrane-bound organelles [8,9]: a proliferative state can be observed in Jurkat T cells at $[2]_{ss} < 0.7\,\mu M$; at slightly higher levels (1–3 μM) cells enter the apoptotic process and, at even higher levels (>3 μM) cells undergo necrosis (Fig. 1).

Abbreviations: AKT, serine/threonine kinase (also known as protein kinase B); AP-1, activator protein-1; Apaf-1, apoptosis protease activating factor-1; ASK1, apoptosis signal-regulating kinase-1; Bad, Bcl-2/$BclX_L$-associated death promoting protein; Bax, pro-apoptotic protein of the Bcl-2 family; Bcl-2, B-cell lymphoma 2: protein with anti-apoptotic properties; $BclX_L$, long form of Bclx: anti-apoptotic protein of the Bcl-2 family; Caspase, cysteinyl aspartic acid-protease; c-Jun, mammalian equivalent of Jun: part of the AP-1 transcription complex; CREB, cAMP response element binding protein; DIABLO/smac, mitochondria-related pro-apoptotic protein: inhibits XIAP; ERK1/2, extracellular signal-related kinase; Fas, CD95: member of the TNF receptor family; GSHST, glutathione *S*-transferase; JNK, c-Jun amino-terminal kinase; MAPK, mitogen-activated protein kinase; MAPKK, MAPK kinase; MAPKKK, MAPKK kinase; MEK1/2, ERK1/2-specific MAPKK; MKK4/7, JNK-specific MAPKK; MSK1, mitogen- and stress-activated kinase-1; NF-κB, nuclear factor of immunoglobulin k locus in B-cells; $ONOO^-$, peroxynitrite anion; *p53*, pro-apoptotic tumor suppressor gene product; PI3-kinase, phosphoinositol 3-kinase; Ras, small G-protein; RNS, reactive nitrogen species; ROS, reactive oxygen species; RSK, pp90 ribosomal S6 kinase; SAPK, stress-activated protein kinase; XIAP, X-linked inhibitor of apoptosis: inhibits the activation of caspase-9

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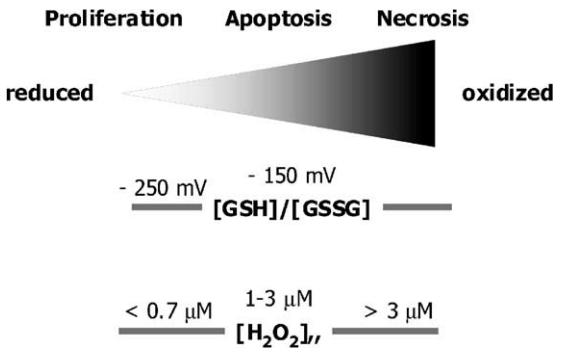

Fig. 1. The cellular redox state, glutathione, and hydrogen peroxide levels. In proliferating cells, the glutathione redox status is approximately −250 mV, whereas, apoptotic cells have a redox status of approximately −150 mV. The steady-state level of hydrogen peroxide in proliferating cells was calculated to be below 0.7 μM increasing to over 3 μM under conditions of necrosis. The glutathione redox potential values and the hydrogen peroxide steady-state levels appear to represent a continuum from a reduced state to an oxidized state as cell progress from cell division to cell death.

Taken together oxidative/nitrosative stress is the shift of the redox status of a cell towards oxidation resulting in the impairment of cellular function and damage to essential biomolecules. The cellular responses to oxidative insults range from survival, repair of damage and the enhancement of cellular antioxidant defences, on the one hand, to cellular senescence and apoptotic or necrotic cell death, at the other extreme. This places intracellular signaling pathways in a key position to sense, interpret, and ultimately determine the outcome following a shift in the redox status, or an oxidative insult. The differential sensitivity of cell types to oxidative and nitrosative stress is a function of the steady-state levels of the oxidant, its chemical reactivity, and the general cellular environment as evaluated by the redox-sensitive components of intracellular signal transduction. These parameters are taken into consideration in this review when addressing the differential effects of dietary flavonoids and the endogenous free radical nitric oxide (NO•) on signal transduction pathways involving mitogen-activated protein kinase (MAPK), and their implications for neurodegeneration (Fig. 2).

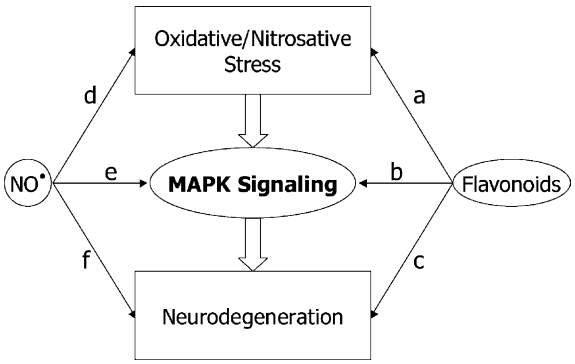

Fig. 2. MAPK signalling in neurodegeneration: influences of flavonoids and nitric oxide. The scheme demonstrates potential sites of action for flavonoids and nitric oxide with respect to their proposed modulatory effects in neurodegeneration as addressed in this review. Flavonoids might (a) directly influence oxidative/nitrosative stress due to their hydrogen-donating properties, (b) specifically act within MAPK signaling cascades, or (c) influence neurodegeneration by other means, for example modulation of GABA-receptor properties or mitochondrial function. Nitric oxide may (d) directly influence the intracellular redox-state, (e) modulate MAPK signaling, (f) alter other cellular functions including mitochondrial function or neurotransmitter release.

2. Nitric oxide and the cellular redox state

The differential sensitivity of cell types to a steady-state level of NO• is not simply a consequence of the amount of NO• produced, but more importantly, the nature of the RNS generated and the prevailing cellular environment. The interplay between several factors within the local microenvironment in which NO• is released is critical in determining which species are generated and whether a cytotoxic or cytostatic response is manifested physiologically. These factors include the cellular redox state, hydrophobicity of the local environment, presence of other reactive mediators, and the nature, bioavailability, and proximity of molecular targets [79].

The main reactions of NO• of biological interest and of potential significance for modulation of signal transduction pathways are (a) its reduction to NO^-(nitroxide ion) (b) its oxidation to NO^+(nitrosonium ion) and (c) radical–radical addition reactions [45]. The former follows general Eq. (1) and occurs with a variety of electron donors yielding the triplet-state NO^- and the radical form of the donor. The oxidation of NO• to NO^+ requires strong oxidants, such as hydroxyl radical (HO•), nitrogen dioxide (NO_2), or peroxynitrite anion ($ONOO^-$). Within the cellular environment, NO^+ exists as a pool of nitrosating species or NO^+-donors, such as HNO_2, N_2O_3, and N_2O_4. Nitrosating species can nitrosate nucleophiles, such as thiols, amines, and aromatics; hence, the overall effect is that of NO• addition reactions (nitrosation or nitrosylation) (Eq. (2)). The third type of reaction, the radical–radical addition includes mainly the reaction of NO• with superoxide anion, which occurs at a diffusion-controlled rate ($1.9 \times 10^{10}\,M\,s^{-1}$) (Eq. (3)) and those with the above-mentioned radicals (HO•, NO_2, $ONOO^-$):

$$NO^\bullet + AH^- \rightarrow NO^- + A^{\bullet -}, \quad (1)$$

$$^\bullet NO \underset{HO^\bullet, NO_2^\bullet, ONOO^-}{\overset{-le^-}{\rightarrow}} NO^+ \underset{RS^-, NH_2, Ar{-}OH}{\overset{addition}{\rightarrow}} RS{-}N{=}O, \quad (2)$$

$$NO^\bullet + O_2^{\bullet -} \rightarrow ONOO^-. \quad (3)$$

The NO• addition reactions (Eq. (2)) accomplished through intermediate formation of nitrosating species and leading to the formation of nitrosothiols are the subject of intensive research: it may be surmised that altering the redox status of critical thiols by NO• may serve a signaling function different from that originating from the reaction of

$NO^{\bullet}$ with the soluble gluanylate cyclase heme and leading to cGMP production. The reaction of $NO^{\bullet}$ with iron ligands, however, may serve as a mechanism of reductive nitrosation, a process involving reduction of the metal by bound $NO^{\bullet}$ (Eq. (4)) followed by nitrosation of a nucleophile (e.g. thiol) by the intermediate complex (Eq. (5)) [68]:

$$NO^{\bullet} + Fe^{III} \rightarrow [Fe^{III} - NO^{\bullet} \Leftrightarrow Fe^{II} - NO^{+}], \quad (4)$$

$$[Fe^{III} - NO^{\bullet} \Leftrightarrow Fe^{II} - NO^{+}] + RSH \rightarrow Fe^{II} + RS\text{–}N{=}O. \quad (5)$$

$NO^{\bullet}$ and derived species can thus react reversibly or irreversibly with a vast array of cellular components, many of which are involved in cell signaling. Reactions mediated by peroxynitrite (e.g. carbonyl formation, nitration of tyrosine and tryptophan, oxidation of methionine, base deamination) typically lead to irreversible damage to proteins, lipids, and DNA and thus, loss of function. This fact, coupled with a biological half-life of seconds, would suggest that peroxynitrite chemistry is not amenable to cell signaling. However, there is a growing body of research arguing for such a role for peroxynitrite [79,119]. Post-translational modification of proteins by *S*-nitrosation, however, is an excellent candidate for cellular signaling. *S*-nitrosation is reversible process, allowing termination of the signal. To reiterate, $NO^{\bullet}$ cannot react with thiols directly, but must proceed via a more redox active form, either a metal–$NO^{\bullet}$ complex or by the formation of nitrosating species [68]. The process of *S*-nitrosation and *trans*-nitrosation is also redox regulated, being very sensitive to GSH and ROS. Nitrosothiols, in particular *S*-nitrosoglutathione, are a bioactive source of NO^{+} prolonging the effective physiological half-life of $NO^{\bullet}$ through controlled decay, and are important in *trans*-nitrosation reactions. This points to an important interplay between the cellular redox state, GSH/GSSG ratio, *S*-nitrosation and the formation of mixed disulfides, also implicated in cell signaling.

3. Flavonoids: chemical and biological implications for their bioactivity

3.1. Flavonoids: antioxidant properties

There has been considerable interest in recent years in the cytoprotective and neuroprotective effects of flavonoids, especially in the context of their modes of action as antioxidants. The electron-donating properties of flavonoids are well defined to explain their antioxidant properties in vitro [19,29,166,171,188]. Structurally important features defining the reduction potential of flavonoids are the hydroxylation pattern, a 3′,4′-dihydroxy catechol structure in the B-ring, the planarity of the molecule and the presence of 2,3-unsaturation in conjugation with a 4-oxo function in the C-ring. Many studies have described the antioxidant efficacy of flavonoids in inhibiting the lipid peroxidation [29,82,171] and other biomolecules such as proteins and DNA [7,20,169] in vitro. Of particular importance to neurodegenerative diseases such as Parkinson's disease is the ability of flavonoids to inhibit peroxynitrite-mediated oxidation of dopamine and peroxynitrite-mediated nitration of tyrosine in vitro by a structure-dependent mechanism involving either the oxidation or nitration of the flavonoid ring system [98,150,151]. In addition, their ability to act as antioxidants in vitro is based on metal-chelating capacity [22,138] and on the quenching of singlet oxygen [203]. However, although flavonoids react rapidly with ROS/RNS in chemical systems in vitro, their reactions in vivo will be dependent on the form that is bioavailable to cells and tissues.

3.2. Metabolism and distribution of flavonoids in mammals and the implication for their bioactivity

Although the pool of data demonstrating the in vitro effects of flavonoids as antioxidants or modulators of protein functions is extensive, only little is known about the antioxidant potential and bioactivity of in vivo flavonoid metabolites. This is surprising since early investigations in the 1950–1960s in mammals already indicated that most of the flavonoids are conjugated and metabolized mainly in the liver or degraded by the colonic microflora (Fig. 3).

More recently, investigations of the sites and mechanisms of metabolism of flavonoids have established that flavonoids can be metabolised in the small intestine. Studies using the isolated rat small intestine have also established that flavonoids are substrates for β-glucosidases [184,192], UDP-glucuronyl transferases [110,111,192], and catechol-*O*-methyl transferases leading to methylation of *o*-dihydroxy catechol structures and that the major metabolites of epicatechin on transfer across the small intestine include *O*-methylated and glucuronidated forms [110] (Fig. 4). This is consistent with observations derived from human supplementation studies identifying *O*-methylated, glucuronidated and sulfated products in the circulation and eliminated in the urine. Metabolism can also occur in the colon and result in extensive modifications including hydrolysis, oxidation, and ring cleavage, forming secondary phenolic metabolites. Thus, the bioactivity of flavonoids in vivo is most likely to derive from their conjugates and metabolites and not necessarily from the ingested dietary forms.

In view of their powerful antioxidant properties in vitro [166], many studies have examined the absorption and bioavailability of the (epi)catechin flavanols, major constituents of read wine, tea, cocoa products, and other plant-derived foods. Recent work by Okushio et al. [147] reported that both the *O*-methylated form and glucuronidated conjugates could be detected in rat urine after oral administration of epicatechin. Furthermore, epicatechin-5-*O*-β-glucuronide and catechin-5-*O*-β-glucuronide were detected in plasma, bile, and urine of rats after consumption of epicatechin and

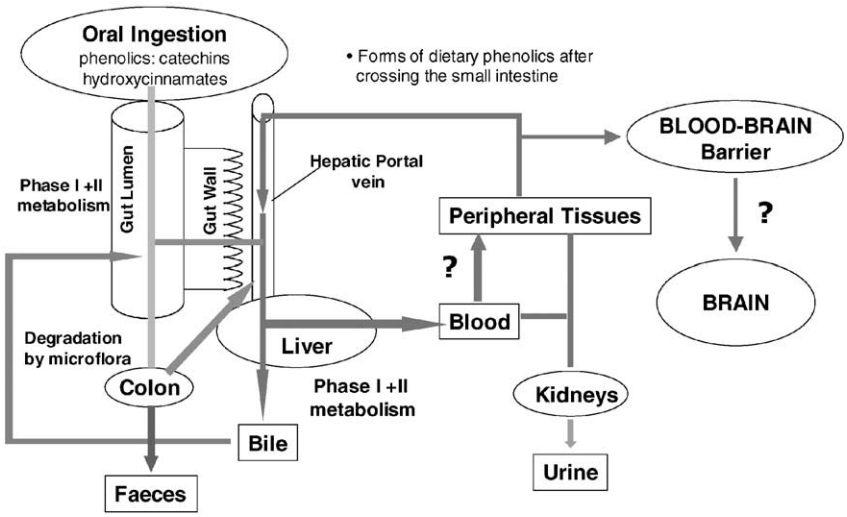

Fig. 3. Possible routes for dietary flavonoids after oral ingestion. Following oral ingestion flavonoids enter the gastrointestinal tract were they may be cleaved from their glycosidic moieties, taken up and undergo further Phase I/II metabolism. The remaining flavonoid aglycones and/or metabolised derivatives enter the portal vein and are transported to the liver were further metabolism will take place. Subsequently, flavonoid metabolites enter the circulation were they may be distributed to the peripheral tissues, perhaps even across the blood–brain barrier, or urinary excreted. Flavonoids, reaching the colon will be excreted or undergo extensive degradation by the colonic microflora. The resulting phenolic compounds may be absorbed.

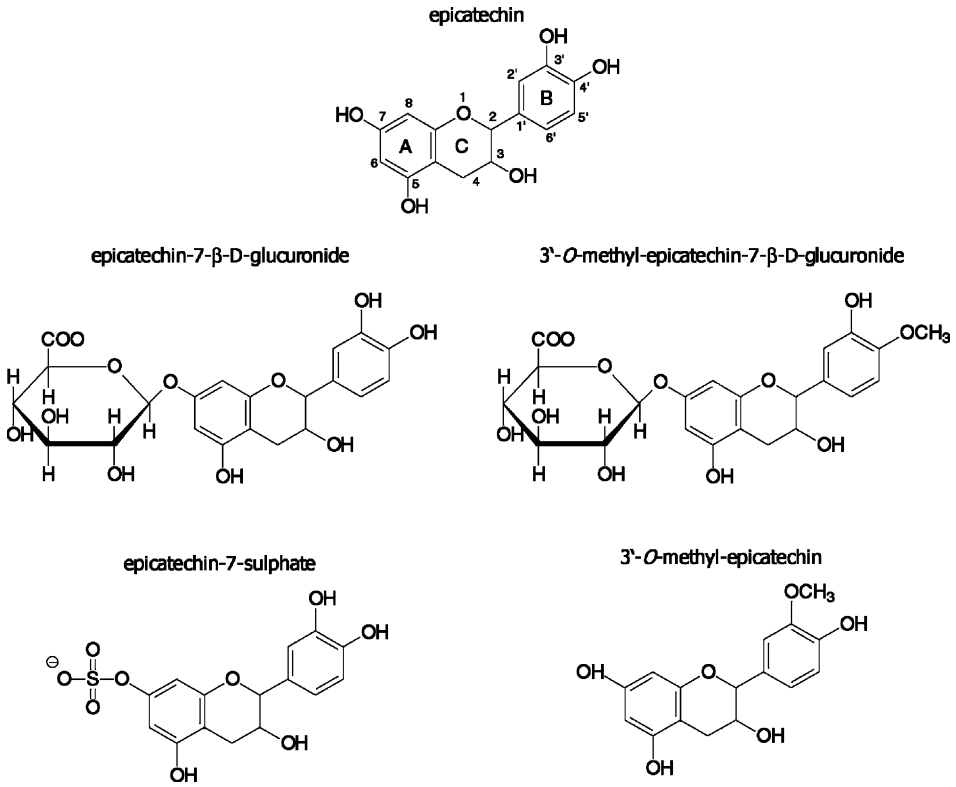

Fig. 4. Structures of potential in vivo metabolites of epicatechin. The major metabolites of epicatechin in vivo are glucuronidated and/or *O*-methylated and sulfated derivatives. Whereas the *O*-methylation of epicatechin always modifies the B-ring, glucuronidation might predominantly occur in five or seven position.

catechin, respectively [78]. In humans, increases in 3′-*O*-methyl-catechin, sulphate, and glucuronide metabolites in plasma were observed after consumption of either red wine or de-alcoholised red wine [56].

There is currently considerable interest in the potential neuroprotective effects of (epi)catechin and its physiologically important metabolites. Thus, the question arises as to whether the in vivo metabolites of epicatechin, including *O*-methylated and glucuronidated derivatives, cross the blood–brain barrier, whether glucuronides have access to the brain due to their polarity or are hydrolyzed prior to uptake, or whether the *O*-methylated compounds are more likely to enter the brain due to their higher lipophilicity. Studies applying radio-labeled epigallocatechin gallate, a tea polyphenol, have demonstrated a wide distribution in mouse tissues, including the brain [198]. This was supported by other investigators describing the detection of the flavonoids hesperetin and naringenin, and naringenin glucuronide, in brain tissue following i.v. or oral administration [155,206].

3.3. Biological and cellular properties

The major emphasis in recent years has concerned the potent in vitro antioxidant effects of flavonoids described in numerous publications (reviewed in [166]). However, flavonoids exhibit various effects on mammalian cells with interesting implications for inflammation [133], cardiovascular disease [81,83] and cancer [58] involving the modulation of redox functions, calcium homeostasis [227], the activity of various enzyme systems, proliferation and differentiation, and the response to a variety of stimuli (reviewed in [134]). The effects of flavonoids are often pictured as beneficial for cell survival, preventive against oxidative insults and anti-carcinogenic. However, the actions of flavonoids are complex and often seemingly antagonistic or paradoxical. For example, while flavonoids are described as antioxidant agents protecting against oxidative insults to cells and apoptosis [11,13,88], others have found flavonoids to be pro-oxidants and -apoptotic [33,212,221]. Thus, it becomes clear that the effects of flavonoids depend on different factors such as the specific compound used, the cell type, concentrations, experimental design, and the general context in which flavonoids are used. Furthermore, data reporting the action of flavonoids might often be observational, failing to explain the molecular/cellular basis of such observations. However, accumulating evidence suggests that flavonoids interact selectively within MAPK signaling cascades [104,107]. This could have important implications with regard to their possible sites of action in neurons since members of the MAPK family are involved in signaling to neuronal survival, regeneration and death [48,136,228]. Indeed, recent reports on oxidative stress in primary striatal neurons demonstrated that flavonoids, in particular epicatechin and kaempferol, are able to attenuate the activation of extracellular signal-related kinases (ERK1/2) and JNK and protect against neuronal cell death [178].

4. MAP kinase signaling

MAP kinase signaling (MAPK) pathways transduce extracellular and intracellular stimuli into cellular responses. In general, MAPK signaling motifs are highly conserved throughout evolution and appear to be essential signal transduction systems in yeast, higher eukaryotes as well as in plants. These responses consist of phosphorylation of cytosolic or nuclear target proteins and activation of transcription factors, which consequently modulate gene expression. Mammals express at least three distinctly regulated groups of MAPKs, which may exist in different isoforms: ERK1/2, c-Jun amino-terminal kinases (JNK1/2/3), and p38 kinases (p38α/β/γ/δ) (reviewed in [34]).

Each MAPKK can be activated by more than one MAPKKK, increasing the complexity and diversity of MAPK signaling. To be activated, all members of the MAPK family require dual phosphorylation of a threonine and tyrosine residue within the catalytic domain by their respective upstream kinase. Hence ERK, JNK, and p38 contain the specific dual phosphorylation motif Thr–Glu–Tyr, Thr–Pro–Tyr, and Thr–Gly–Tyr, respectively. Besides the upstream kinases, the activation of MAPKs critically depends on the activity of a special family of dual specificity phosphatases the MAP kinase phosphatases (MKPs) that inactivate MAPK and, therefore, play an important role in the dynamics of MAPK signaling [34].

Active MAPKs function as modulators for differentiation, proliferation, cell death, and survival. Commonly, the activation of ERK1/2 has been linked to cell survival, whereas that of JNK and p38 (also called the stress-activated protein kinase (SAPK)) has been associated with apoptosis. This perspective is an oversimplification and the actual roles are highly dependent on the cell type, the state of cell development, the kind of stimulus and the context of stimulation.

Activation of ERK1/2 can lead to the phosphorylation of a wide array of potential targets in cytosol or nucleus. For example active ERK1/2 can activate transcription factors [215] and phosphorylate specific effector kinases—the MAPK-activated protein kinases (MAPKAPKs) such as the mitogen- and stress-activated kinase-1 (MSK1) or the pp90 ribosomal S6 kinase (RSK) [197]. RSK phosphorylates the Bcl-2 family member Bad, thereby inhibiting its pro-apoptotic activity [18]. RSK and MSK1 are also potent activators of the cAMP response element binding protein (CREB), a transcription factor for Bcl-2 and, therefore, an important factor for cell survival [51]. This would suggest that active ERK1/2 plays an important role in pro-survival signal transduction processes. Indeed different studies clearly indicate that under certain conditions the activation of ERK1/2 is essential to neuronal survival [18,218]. Furthermore, the activation of Ras, an initiator of the ERK1/2 signaling cascade, has been shown to also activate the phosphoinositol 3-kinase (PI3-kinase)/AKT pathway, another important survival pathway in neurons, demonstrating possible interlinks between different survival signals [129].

Interestingly, the protective effects initiated by Ras activation involve the suppression of pro-apoptotic signals such as the tumor suppressor protein p53 and the Bcl-2 family member Bax and are less potent if either the ERK1/2 or the PI3-kinase/AKT pathway is selectively inhibited [129]. Thus, this describes the outcome of a signal transduction process as the sum of different actions towards the same target and underlines the importance of interlinks between signaling cascades in promoting and enhancing specific signals.

However, the actual role of ERK1/2 seems to be dependent on various parameters, since inhibition of ERK1/2 activation during focal ischemia [3], oxidative stress [198], and a model for hippocampal seizures [140] attenuated neuronal death and cellular injury, indicating a pro-death singling role for ERK1/2. In addition, the inhibition of ERK1/2 activation has been demonstrated to protect a mouse neuronal cell line and rat primary cortical neurons from oxidative stress-induced neurotoxicity [175]. Furthermore, nitrosative stress-induced apoptosis in a hippocampal model of glutamate-mediated neurotoxicity has been shown to involve activation of ERK1/2 [228].

Active JNKs have a wide range of potential phosphorylation targets in the nucleus as well as in the cytoplasm. Nuclear substrates for JNKs are transcription factors such as c-Jun [1,75,92], ATF-2 [92], and ELK-1 [30]. As far as is known JNKs are the only kinases capable of phosphorylating c-Jun in vivo. c-Jun is part of the activator protein-1 (AP-1) transcription factor, which either exists as Jun homodimer or as a Jun/Fos heterodimer [95]. The transcriptional activity of the AP-1 complex is increased [95] upon phosphorylation of Ser-63 and -73 of c-Jun by JNK [160]. In addition to c-Jun, JNKs also phosphorylate other AP-1 proteins, including JunB and JunD [25,95]. The regulatory effect of JNKs on AP-1 transcription might not only be due to phosphorylation of Jun but may also involve JNK-regulated ubiquitin-mediated degradation of AP-1 proteins [66,67]. JNKs appear to be essential in cytokine and stress-induced activation of AP-1 but are not required for AP-1 activation in response to other stimuli. Cytosolic substrates for JNKs include cytoskeletal proteins, the tumor suppressor protein p53 [24,65], the mitogen-activated kinase activating death domain protein (MADD) [232], glucocorticoid receptors [167], Bcl-2, and $BclX_L$ [220], neurofilaments, tau [84,165] and STAT-3 a member of the signal transducer and activators of transcription (STAT) family [122,207].

Although JNK activation is often associated with cellular injury and degeneration it also plays an important part in embryonic morphogenesis, memory formation, and immune defence. The pro-apoptotic action of JNKs in neurons was initially investigated in neuronal cell death following the withdrawal of neurotrophic factors [218]. It was found that JNK activation contributed to the apoptotic response and that JNK-mediated apoptosis was suppressed by activation of survival pathways. Studies in mice utilizing the disruption of the neuronal gene *JNK3* confirmed the role of JNK in stress-induced neuronal apoptosis by demonstrating that JNK3-knockout mice were developmentally normal but resistant to excitotoxin-induced neuronal apoptosis [222]. These findings were supported by observations in mice with a mutation in the c-*Jun* gene that altered the JNK phosphorylation sites of c-Jun and led to a resistance against kainic acid triggered death of hippocampal neurons [15]. Further support arose from data obtained using dominant-negative c-Jun mutants, which reduced sympathetic neuronal death following NGF withdrawal [214]. A pro-apoptotic function has also been suggested for JNK activation in NGF-withdrawal-induced neuronal death [59] in a hippocampal model of Huntington's disease [124] and in beta-amyloid-induced neuronal apoptosis [204]. However, the apoptotic process does not occur in JNK-knockout mice [222] or mice expressing a mutant form of c-Jun lacking the JNK phosphorylation site [15]. In addition, the indirect inhibition of JNK by CEP-1347 protected neuronal PC12 cells and sympathetic neurons in vitro from death following trophic factor withdrawal, beta-amyloid exposure, UV-radiation and oxidative stress [126,205]. Furthermore, CEP-1347 protected nigral neurons in vivo against MPP^+-induced neuronal death [173] implicating again the involvement of JNK in neurodegeneration. Moreover, JNK activity and apoptosis in cerebellar granule neurons was enhanced following inhibition of the pro-survival pathway PI3-kinase/AKT [185]. Finally JNKs seem to be indirectly involved in other apoptotic pathways by enhancing transcription of death receptors such as Fas-L [62] or by activating and stabilizing the p53 protein [24]. Together these data strongly support JNK involvement in apoptosis signaling in neurons.

A recent review by Davis [48] on the role of JNK in apoptosis suggested a mechanism of JNK-dependent apoptosis involving the mitochondria and caspase-3. This hypothesis is based on observations in primary murine embryonic fibroblasts (MEF) lacking the genes for *JNK1* and *JNK2* (JNK null; no JNK1/2 protein/activity). These JNK null MEF exhibit profound defects in stress-induced (UV-radiation, DNA-alkylation, translational inhibition) apoptosis [204]. The defect in the execution of apoptotic cell death was caused by the failure to initiate JNK-induced cytochrome *c* release from the mitochondria [204]. This malfunction is significant since it is critical for the subsequent sequential activation of Apaf-1 [120], the initiator-caspase caspase-9 [76] and finally the effector-caspase caspase-3 [217] all of which are essential in the execution of apoptosis. Tournier et al. [204] suggested that the apoptotic response is suppressed in JNK null MEF due to the absence of JNK, which is needed to initiate the apoptotic cascade (Fig. 5). However, it is not clear yet by which molecular mechanism JNK mediates the release of cytochrome *c* from the mitochondria. Although a c-Jun-activated transcription seems possible (Fig. 5) [15,214], it is not required for UV-induced apoptosis [204]. Several studies point to the JNK mediated in vitro phosphorylation/inactivation of the mitochondria-associated anti-apoptotic proteins Bcl-2 and $BclX_L$ [40,61,94] as a

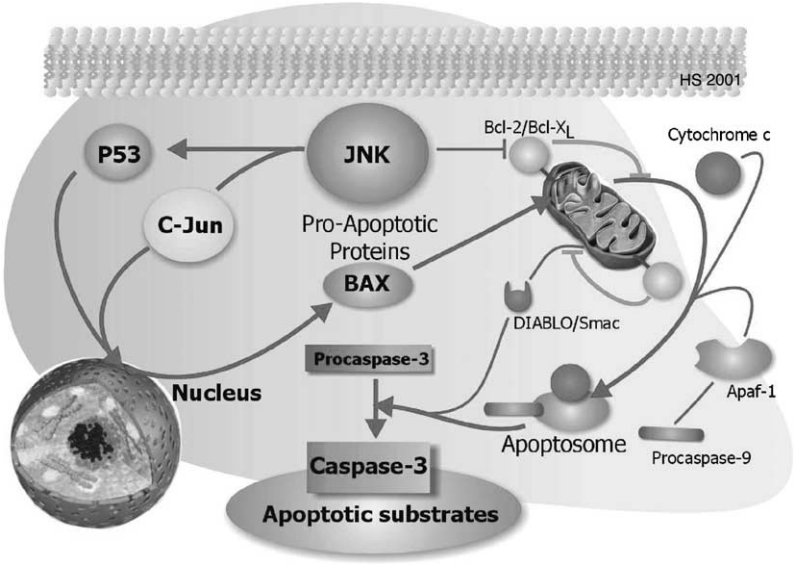

Fig. 5. The possible involvement of JNK signaling in the execution of cell death. Once activated JNK can phosphorylate p53, which in term stabilizes and activates this apoptotic mediator resulting in the suppression of the anti-apoptotic protein Bcl-2 and the induction of the pro-apoptotic protein Bax. Active JNK may also influence the expression of other pro-apoptotic molecules via the c-Jun/AP-1-mediated regulation of their expression. The proposed function for active JNK as mediator of the phosphorylation of the mitochondria-associated proteins Bcl-2 and $BclX_L$ will alter their anti-apoptotic functions and may subsequently result in the release of cytochrome *c* or other apoptotic factors such as DIABLO/smac from the mitochondria. The release of cytochrome *c* will in turn promote the formation of the apoptosome, subsequently leading to the activation of caspase-3 one of the major executors of apoptosis.

possible mechanism, since Bcl-2/$BclX_L$ are known to regulate cytochrome *c* release (Fig. 5). Others proposed an as yet unknown adaptor protein as the mediator for JNK actions. Further studies are needed to substantiate this hypothesis and establish the molecular mechanisms.

In summary, it is becoming increasingly clear that JNK activation is involved in apoptotic processes in vivo and in vitro either by direct effects of JNK mediated c-Jun phosphorylation, the adverse effects on survival pathways or the indirect effects on cell function via other mediator systems. But apoptosis is not the inevitable outcome of JNK signaling. In fact, the overall cellular outcome of a signal transduction process might rather be determined by the sum of both pro-survival and -apoptotic signaling pathways.

4.1. MAPK signaling under oxidative stress

Oxidative stress seems to be a major stimulus for MAPK signaling cascades with cell survival or death as a possible consequence. The observation that multiple pathways are sensitive to alteration in intracellular ROS/RNS concentrations indicates that this might represent a common cellular pathway to signal a large diversity of different stressful stimuli. Accordingly a large number of redox-responsive transcription factors and genes have been identified [5].

Oxidative stress has been shown to contribute to the neuropathology of a number of neurodegenerative disorders [77], including Alzheimer's disease [14], Parkinson's disease [231] and Huntington's disease [4], as well as being implicated in neuronal loss associated with age-related cognitive decline [64,161], cerebral ischemia/reperfusion injuries, seizures [44,148], trauma, and neuroinflammation [130,131].

High levels of ROS and RNS can disrupt the normal redox state and shift cells into the state of oxidative stress, hallmarked by intracellular increases in products of lipid peroxidation, hydrogen peroxide, and elevated damage to other biomolecules. It is often stated that the damage to important biomolecules such as proteins and DNA as a result of oxidative stress leads to cell injury or death. Importantly, this oversimplification ignores a number of stress response mechanisms that cells have developed to coordinate reactions that ultimately determine the outcome following an oxidative insult. Among the main stress signaling pathways or central mediators in stress response to oxidative insults are the MAPK cascades, the PI3-kinase/AKT, the nuclear factor κB (NF-κB) signaling system, p53 activation and the heat shock protein response. In summary, accumulating evidence seemingly indicates a central role for the MAPK signaling cascade in neuronal survival and death during the development and the aging of the CNS, as well as in the pathology of neurodegenerative diseases [48,136,226].

Oxidative stress seems to be a major stimulus for MAPK signaling cascades. However, this picture would appear to be too simplistic since a shift of the overall reductive environment that most cells maintain under physiological conditions towards reduction also leads to the activation of MAPK signal transduction with cell survival or death as a possible consequence.

Growth factor receptors have been shown to undergo enhanced phosphorylation in response to oxidative insults like hydrogen peroxide or UV-radiation initiating ERK1/2 activation [115,116,210]. This is consistent with demonstrations of the mitogenic effects of low concentrations of hydrogen peroxide [23]. However, the inhibition of ERK1/2 activation has been shown to protect a mouse neuronal cell line and rat primary cortical neurons form oxidative stress-induced neurotoxicity [1] demonstrating a possible involvement in cell death. The JNK signaling cascade has been reported to be activated by a wide range of different oxidants/reductants including hydrogen peroxide [135,218], lipid peroxidation products [6,191], different types of radiation [63], modulators of intracellular glutathione status [145], peroxynitrite [71], glutamate [180], dithiothreitol, and nitric oxide [152]. Although the links between redox status and MAPK signaling have been known for some time, data demonstrating the molecular basis of such links and identifying the sensors in this redox response are few.

Accumulating evidence indicates that members of the MAPK family or their upstream or downstream partners have such redox-sensitive motifs. For example, JNK itself exhibits a redox-sensitive cysteine residue that is not present on ERK or p38 [153]. An additional mechanism in JNK redox regulation is its binding to redox-sensitive proteins such as glutathione *S*-transferase (GSHST) [2]. It has been shown that under non-stressed conditions, JNK is associated with GSHST resulting in the inhibition of JNK activity, but that GSHST dissociates from JNK following UV-radiation or hydrogen peroxide treatment [2,224,225]. Forced expression of GSHST decreased JNK activity, increased c-Jun ubiquitination and reduced c-Jun-mediated transcription [2]. In addition, GSHST expression in NIH 3T3 cells lead to the attenuation of hydrogen peroxide-induced JNK activation as well to an increase in ERK1/2 activity [224,225]. Similarly GSHST and the redox regulatory protein thioredoxin (Trx) bind under non-stressed conditions to the apoptosis signal-regulating kinase-1 (ASK1) [39,170,225], an upstream activator of JNK, and inhibit JNK activity. However, oxidative insults cause the dissociation of the Trx-ASK1 and GSHST complex and the subsequent activation of JNK [39,170,225].

Ca^{2+}-Homeostasis is another important mediator/regulator of oxidative stress-induced signaling, since both ERK and JNK are sensitive to changes in intracellular Ca^{2+}-concentrations [60,102,142]. This Ca^{2+}-sensitivity of ERK/JNK signaling might play a role in oxidative stress-induced signaling events, since it has been demonstrated that oxidative insults often influence normal Ca^{2+} homeostasis in cells. With regard to ERK1/2, Samanta et al. [172], demonstrated for the first time that hydrogen peroxide induced activation of ERK1/2 in primary neurons is strictly dependent on extracellular calcium. Ca^{2+}-dependent MAPK signaling has also been suggested to play a role in glutamate receptor-mediated neuronal stress [156]. The influx of Ca^{2+} into the cytosol from the extracellular space or from intracellular stores following a stressful stimulus can activate the Ca^{2+}/calmodulin kinases, which in turn can stimulate the activation of all three MAPKs [142]. The effect of ROS/RNS on MAPK activation is complex and occurs at multiple levels and further results are needed to elucidate the molecular basis of these interactions.

5. Nitric oxide and MAPK signaling

The role of $NO^{\bullet}$ in cellular signaling was first identified in the vascular system with respect to the regulation of smooth muscle contraction, important for vascular tone [85]. $NO^{\bullet}$ is a potent activator of soluble guanylate cyclase through binding to the essential heme Fe^{2+} [85]. This leads to elevated cGMP levels and increased activity of cGMP-dependent kinases and phosphatases. $NO^{\bullet}$ has been identified as a novel neural messenger, promoting Ca^{2+}-dependent neurotransmitter release from synaptic storage vesicle [70]. $NO^{\bullet}$ modulates exocytosis through cGMP-dependent protein phosphorylation cascades following the classic activation of soluble guanylate cyclase [70]. The high diffusibility of $NO^{\bullet}$ makes it an ideal retrograde signal for the two forms of synaptic modulation required for learning and memory, namely long-term potentiation in the hippocampus and long-term depression in the cerebellum [70].

Besides a role in differentiation and synaptic plasticity, $NO^{\bullet}$ has also been implicated in neuronal apoptosis, and consequently, in neurodegenerative diseases, specifically when $NO^{\bullet}$ production is increased to toxic levels [49,64,77,123,202]. In particular, $NO^{\bullet}$ has been linked to the phenomenon of excitotoxicity involving the over-stimulation of the NMDA receptor by glutamate, subsequently triggering a strong intracellular accumulation of Ca^{2+} [141]. This Ca^{2+} overload in neurons leads to a substantial increase in the activity of Ca^{2+}/calmodulin-dependent nitric oxide synthase (neuronal NOS) resulting in a high intracellular concentration of $NO^{\bullet}$, which has been identified as a mediator of glutamate-induced neuronal death [49]. Furthermore, microglia activation during neuroinflammation is associated with induction of inducible nitric oxide synthase (iNOS) expression leading to a large and sustained $NO^{\bullet}$ production, which also appears to be causally linked to neuronal apoptosis and neurodegeneration [100,121].

On the contrary, there is growing evidence for cGMP-independent $NO^{\bullet}$-mediated cell signaling towards neuronal survival. Depending on its intracellular concentration and the overall cellular context, $NO^{\bullet}$ has been implicated in mechanisms protecting against stress-induced cell injury. The GTP-binding protein Ras is an intermediate in the transduction of signals from membrane receptor tyrosine kinases to the stimulation of MAP kinases [176] (Fig. 6). Ras appears to be a common *signaling* target of reactive free radicals, including $NO^{\bullet}$, and agents that modulate the cellular redox status, *such as* H_2O_2 and GSH [115]. $NO^{\bullet}$ activates Ras by *S*-nitrosation of a highly conserved cysteine residue, leading to GDP/GTP exchange and downstream signaling [114].

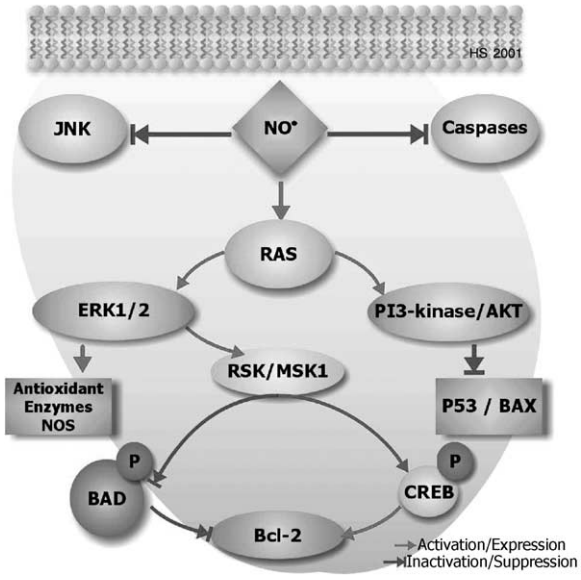

Fig. 6. Potential anti-apoptotic mechanisms mediated by nitric oxide. The nitrosation of Ras mediated by nitric oxide potentially leads to the activation of ERK1/2 or the PI3-kinase/AKT pathway resulting in the suppression of pro-apoptotic proteins such as p53 and Bax or in the upregulation of antioxidant enzymes. Furthermore, Ras activation might mediate the phosphorylation/activation of the CREB, a transcription factor for Bcl-2 and the phosphorylation/inactivation of the pro-apoptotic protein Bad. In addition, the NO•-mediated nitrosation of JNK and caspases may also contribute to the anti-apoptotic properties of NO•.

Thus, it has been proposed that this cysteine reside may represent an important molecular redox trigger, whereby cells can respond to the ambient redox status. Indeed, through Ras activation, NO•-related species can modulate the activity of all three MAPKs [114,115,152]. For example, activation of ERK1/2, as discussed earlier, may lead to the phosphorylation/inactivation of the pro-apoptotic protein Bad, the expression of anti-apoptotic proteins such as Bcl-2 and the suppression of pro-apoptotic proteins such as Bax (see above) (Fig. 6). In contrast, however, NO• can directly suppress JNK activation via *S*-nitrosation of a redox-sensitive residue that is not present on ERK or p38 [152,190], thereby supporting pro-survival mechanisms (Fig. 6). Alternatively, c-Jun, the substrate for JNK, can be directly *S*-nitrosated by NO• [103]. In addition, signaling by kinase-catalyzed phosphorylation can be counteracted by phosphatase-catalyzed de-phosphorlyation, both systems representing targets for NO•. For example, the protective effect of NO• against tumor necrosis factor-α-induced apoptosis in endothelial cells involves prolonged activation of ERK1/2 following inhibition of MAPK phosphatase-3 by NO• [103].

Ultimately, *S*-nitrosation can thus, regulate several redox-sensitive transcription factors including NF-κB, AP-1, Sp-1, and p53, and increase levels of active c-Fos and c-Jun [127]. Indeed, the expression of some enzymes implicated in antioxidant defences such as the enzymes for glutathione synthesis [235] and heme oxygenase [37] exhibit ERK1/2 dependency in the regulation of their expression (Fig. 6). Furthermore, the transcription of Cu/ZnSOD is regulated by the transcription factor ELK-1 [35] and the promoter for MnSOD expression contain binding sites for Sp1, AP-1, and CREB [47,181], all of which have been linked to regulation by ERK1/2 [18,182] (Fig. 6).

In turn, MAPK are also involved in the regulation of the gene expression of all three NOS isoenzymes. The expression of iNOS (NOS-2) is regulated by all three MAP kinase pathways in a variety of cell types [16,32,46,93,108,189]. For example, JNK and ERK1/2 pathways are necessary for lipopolysaccharide (LPS)- and interferon-γ-stimulated iNOS expression in mouse macrophage cells, possibly via α-tumor necrosis factor secretion, whereas p38 inhibited induction [31]. The induction of endothelial NOS (eNOS, NOS-3) by estrogen, fibroblast growth factor or epidermal growth factor in endothelial cells involves the Ras–ERK pathway [38,233]. eNOS is phosphorylated, and thus activated, by the serine/threonine protein kinase AKT, which is recruited to the cell membrane by PI3-kinase as an anti-apoptotic mechanism in the response of endothelial cells to shear stress [54]. In the case of neuronal NOS (nNOS, NOS-1), the induction of mRNA, protein expression and activity by nerve growth factor (NGF) in PC12 cells involves activation of the Ras–ERK1/2 pathway, initiating a kinase cascade proceeding from Raf via MEK to ERK [177].

In this regard, it is very interesting that several studies have shown that specific flavonoids can suppress the induction of iNOS gene and protein expression, and NO• production by cytokines and endotoxins in mouse macrophage RAW 264.7 cells [99,109,152]. The mechanism did not include a direct inhibitory effect on enzyme activity, but rather the modulation of cell signaling pathways necessary for NOS gene expression. This has potentially important implications for the role of microglial iNOS gene expression and neuroinflammation in neurodegenerative diseases. Taken together, it becomes increasingly clear that a potential feedback loop exists between NOS, NO• and MAPK—all of which may be modulated by flavonoids—promoting cross-talk between signaling pathways and thus, influencing a variety of different cellular function, especially with regard to cell survival and cell death.

6. Apoptosis, mitochondrial function and MAPK signal transduction

There is a growing recognition that neuronal cell death characteristic of neurodegeneration is primarily a result of apoptosis rather than necrosis [202,228]. The molecular mechanisms underlying oxidative stress-induced neuronal damage are emerging and appear to involve an apoptotic mode of death in which ERK1/2 [175,196] and JNK [48,136,226] have been strongly implicated. Furthermore, there is strong evidence for involvement of mitochondrial

defects in neurodegenerative diseases, with neuronal cell death arising directly from mitochondrially generated ROS, ATP depletion or activation of the mitochondrial-dependent apoptotic pathways [48,202,226]. Apoptosis, or programmed cell death, is an active, controlled process involving multiple signaling cascades, expression of genes and the modulations of mitochondrial functions. The precise mechanism of apoptosis is complex and depends on the pro-apoptotic stimuli, cell type, redox status of the cell and the overall context of cellular functions and should be seen as the sum of pro-apoptotic and anti-apoptotic signals. Initially apoptosis was believed to occur independently of mitochondrial factors. Now it is recognized that mitochondria play a central role in oxidative stress-induced apoptosis [74] since they contain cytochrome *c* and other pro-apoptotic factors essential in Fas receptor-independent apoptosis [120,204]. The emerging view is that pro-apoptotic stimuli result in a transient collapse of the mitochondrial transmembrane potential, which is temporally correlated with opening of the permeability transition pore and the release of apoptosis-initiating factors (AIF) such as cytochrome *c* and DIABLO/smac into the cytosol, subsequently committing the cell to die by mechanisms, involving the Apaf-1-mediated activation of the executing caspase caspase-3 [74,80,202]. Thus, blocking the release of AIF might be the key to prevent oxidative stress-induced apoptosis. However, other investigators reported apoptotic processes involving the depolarization of the mitochondria without translocation of cytochrome *c* to the cytosol [74,101]. This indicates additional mechanisms leading to the recruitment of apoptosis-executing caspases and cell type specific differences in the mechanism of apoptosis. Taken together, blocking mitochondrial permeability transition (mPT) might be a more general feature than cytochrome *c* release in preventing apoptotic processes. Indeed, the anti-apoptotic protein Bcl-2 is believed to protect against apoptosis by preventing mPT and the opening of the mPT pore (mPTP). Bcl-2 is concentrated in the mitochondrial outer membrane, where its interactions with other members of the Bcl-2 family (Bax, Bad, $BclX_L$) or with proteins of the mPTP are believed to be essential for the anti-apoptotic effects of Bcl-2 [40,61,80].

In the context of oxidative stress-induced apoptosis, caspase inhibitors have been shown to block 4-HNE-induced activation of JNK [27] and reduce oxidative stress-mediated neuronal death [96,178]. Interestingly, in endothelial cells oxLDL-induced apoptosis correlated with the downregulation of the cellular caspase inhibitor FLIP (FLICE-inhibiting protein; FLICE: Fas-associated death domain homologues ICE-like protease) without impacting on the expression of members of the Bcl-2 family ($BclX_L$, Bcl-2, Bax) [174]. In addition, antioxidants and thiol reductants and, under some conditions ROS, can block or delay apoptosis. This places the cellular redox state as an important determinant in apoptotic signal transduction. In fact, the burst of mitochondrial O_2^- generation associated with apoptosis has been linked to cytochrome *c* release from mitochondria, and can be prevented by Bcl-2 over-expression [26]. The vectorial release of mitochondrial O_2^- (more correctly, H_2O_2) to the cytosol may, in turn, be important in cell signaling [143], e.g. activation of MAPK such as JNK [135,218]. Indeed, a wealth of data on apoptosis is accumulating and emphasizing the potential role of the MAPK signaling cascade and mitochondrial function. The MAPK signal transduction could be regarded as a potential cross-link between the different apoptotic pathways. For example, JNK has been proposed to modulate the release of pro-apoptotic factors from the mitochondrion (Fig. 5) and stabilizes the pro-apoptotic p53, which in term may impairs mitochondrial functions thorough the expression of p53AIP1 and a subsequent permeability transition [144]. On the contrary p53 might activate JNK via the MEKK4 involving the p53-mediated expression of the growth arrest and DNA damage-induced protein (GADD45) [201]. Furthermore, the JNK mediated mitochondrial dysfunction can lead to caspase-3 activation (Fig. 5) but on the other hand caspases may activate JNK via MEKK1 [183].

Brookes et al. [21] observed that $NO^\bullet$ modulated cytochrome *c* release from mitochondria in a concentration-dependent manner. At low concentrations, $NO^\bullet$ prevented Ca^{2+}-induced cytochrome *c* release, involving the dissipation of the electrochemical gradient, with a concomitant prevention of intramitochondrial Ca^{2+} accumulation and a closing of the PTP. The primary event involved an inhibition of O_2 consumption, consistent with the fact that $NO^\bullet$ can reversibly inhibit cytochrome *c* oxidase [41]. However, at high concentrations $NO^\bullet$-induced cytochrome *c* release, most likely through peroxynitrite formation [21].

Besides preventing cytochrome *c*-dependent activation of caspase-3, $NO^\bullet$ may also upregulate anti-apoptotic proteins, such as heat shock protein-70 and Bcl-2 via Ras activation (Fig. 6). In addition, the activation of Ras by $NO^\bullet$ as discussed earlier has important anti-apoptotic implications involving either the RSK/MSK1-mediated inhibition of Bad and expression of Bcl-2 as well as the suppression of p53 and Bax (Fig. 6). Furthermore, *S*-nitrosation modulates the activity of other proteins involved in cell death/survival processes such as caspases. Li et al. [120] demonstrated that $NO^\bullet$ can inhibit seven members of the caspase family directly via *S*-nitrosation. The latter include caspase-1, -2, -3, -4, -6, -7, and -8, i.e. representatives from each of the tentative three subfamilies of caspases. On the contrary, it should be noted that through inhibition of O_2 consumption, $NO^\bullet$ can modify electron flux through discrete sections of the respiratory chain. This leads to enhanced mitochondrial O_2^- generation, mainly due to auto-oxidation of ubiquinol [157–159]. Thus, it can be argued that the mitochondria can transduce a nitrosative signal into an oxidative signal, consistent with the proposed role of mitochondrial ROS in cytosolic signaling pathways [143].

However, it remains to be clearly established what intracellular concentrations of $NO^\bullet$ are required for the intracellular nitrosation of proteins and whether or not these

concentrations are relevant under physiological/pathological conditions.

7. Flavonoids: neuroprotective agents in vivo and in vitro?

Data on the anti-apoptotic effects of flavonoids in the context of the CNS or CNS-derived cells are starting to accumulate but not as extensively as for other cells or tissues. Recent epidemiological and dietary intervention studies in humans and animals suggest that flavonoids may play a useful role in preventing neurodegeneration, especially age-related cognitive, motoric, and mood decline and protect against oxidative stress as well as cerebral ischemia/reperfusion injuries. Studies in humans using flavonoid-containing plant extracts, such as *Ginkgo biloba*, or pure flavonoid preparations demonstrate positive effects on cognitive function and memory performance in healthy volunteers from all age groups [42,97,213] as well as in patients with age- or Alzheimer' disease-associated dementia [128,146,208]. Watanabe et al. [211] reported that *G. biloba* supplementation in mice had neuromodulatory effects as indicated by a more than three-fold increase of mRNA expression for neuronal tyrosine/threonine phosphatases-1, microtubule-associated tau, prolactin, different calcium, and chloride channels as well as transthyretin. Flavonoid-associated antioxidant interventions have also been proposed to be beneficial in hypoxia/ischemia, seizures, Parkinson's disease, increased survival in brain cancers, and general age-related neurodegeneration [50,73,89,125]. However, since the flavonoids used in these studies were given as food extracts or preparations containing other potential bioactive or antioxidant components rather than as a pure flavonoid fraction, the results may not reflect solely the effects of flavonoids. Furthermore, the mechanisms of flavonoid actions remain speculative and may involve the antioxidant properties, the modulation of receptors and calcium homeostasis or a combination thereof [89].

In vivo studies in animals demonstrate protective effects of the flavonoids epicatechin and quercetin against ischemia/reperfusion-induced neuronal injury [86,187]. Furthermore, flavonoids showed protective effects against an increase in brain lipid peroxidation following Vitamin E deprivation [219], attenuated neuronal damage induced by ethanol administration [113,199] and reduced *O*-ethyl-*S*,*S*-dipropyl phosphorodithioate-induced neurotoxicity in mice [163]. Interestingly, the oral administration of a catechin-containing antioxidant preparation significantly increased the life span of senescence-accelerated mice [112] and increased SOD activity in the mitochondrial fraction of the striatum and the midbrain, decreased products of lipid peroxidation in cortex and cerebellum, and attenuated the iron(II) chloride-induced formation of markers of DNA damage in the cortex of aged rats [106]. The authors of this study relate the observed effects mainly to the rather unspecific antioxidant activity of catechin and other compounds contained in the preparation and fail to give further insights in the mechanisms related to the increase in mitochondrial SOD activity. Other investigators reported an attenuation of age-related decline in cognitive function and loss of behavioral deficits following long-term dietary supplementation with anthocyanidin-rich foods or plant extracts [90,91,216]. The investigators used several neuronal and behavioral parameters including dopamine-release, GTP-activity, calcium buffering in striatal synaptosomes, rod walking tasks and the water maze performance to elucidate possible mechanisms involved [90,91].

In vitro, observations in neuronal PC12 cells demonstrated the protective effects of *G. biloba* extract against hydrogen peroxide-mediated [209] or beta-amyloid-induced neuronal death [186,223]. Other authors reported beneficial effects of *G. biloba* extract against beta-amyloid-mediated neurotoxicity in primary hippocampal neurons [11,57] and implicated antioxidant activities and the modulation of intracellular calcium levels in PC12 cells [209] as possible mechanisms of action. Other lines of research focused on the binding of flavonoids such as apigenin, naringenin, kaempferol, quercetin-3-*O*-glucoside and others to benzodiazepine binding sites [149] of different receptors including the GABA-A-receptor [52,132] and adenosine receptors [139], and investigated their anxiolytic potential [149] as a possible mechanism of action in the CNS. Bastianetto et al. report that antioxidant effects are not the only mechanism of flavonoid-mediated protection against neuronal death and show attenuation of $NO^{\bullet}$-induced activation of protein kinase C (PKC) by *G. biloba* [12] and resveratrol [13] to be partially involved in the protection against neurotoxicity.

However, the precise mechanisms by which flavonoids exert their neuroprotective actions in vivo and in vitro are presently unclear and it is only recently that experimental evidence is emerging suggesting that the protective properties of these compounds may be mediated via the modulation of signal transduction pathways [178]. For example, accumulating evidence suggests that flavonoids can interact selectively within MAPK signaling cascades in non-neuronal models [104,107]. This could have important implications with regard to their possible sites of action in neurons since members of the MAPK family are involved in signaling to neuronal survival, regeneration, and death [48,136,226].

It has been shown recently that pre-treatment with low micromolar concentration of epicatechin and kaempferol strongly protected against oxLDL-induced death in primary striatal neurons [179] involving annexin-V binding, caspase-3 activation, and DNA-fragmentation [178]. Interestingly, the flavonoid pre-treatments did not prevent the oxLDL-mediated increase in intracellular oxidative stress but potently inhibited the oxLDL-induced activation of JNK, c-Jun and caspase-3. Furthermore, ascorbic acid did not exert strong protection even when used in concentrations

10 times higher than flavonoids and the in vivo metabolite of epicatechin, 3′-*O*-methyl-epicatechin, a compound with less than half the hydrogen-donating antioxidant activity of epicatechin [194] was found here to have the same ability to attenuate oxLDL-mediated neuronal death. These findings indicate that flavonoids might exert their neuroprotective effects seemingly independent of their classical hydrogen-donating capacity and demonstrate for the first time a possible involvement of flavonoids in neuronal MAPK signaling under oxidative stress. It might be speculated that potential targets of flavonoids modulation of cellular responses to oxidative stress might occur by direct interactions with proteins of signal transduction pathways, by affecting the intracellular calcium homeostasis or influencing mPT. In this context, it is interesting that the chemical structures of various pharmacological inhibitors of intracellular signaling cascades, including the MEK inhibitor PD98059 and the PI3-kinase inhibitor LY294002, are closely related to the basic structure of flavonoids.

As discussed earlier, the mitochondria play a central role in oxidative/nitrosative stress-induced apoptosis and the prevention of mPT and/or the release of AIF such as cytochrome *c* may exemplify potent means to counteract apoptotic neurodegeneration. Speculatively, the flavonoid-mediated inhibition of apoptosis might occur by a variety of means: firstly, by blocking the activation of JNK. This might take place upstream of JNK influencing one of many MAPKKK activating proteins involved in transducing signals to JNK. Secondly, at the level of maintaining the calcium homeostasis shown to be implicated in MAPK activation [60,87,234] and NOS activity. Thirdly, flavonoids might modulate the mPT believed to be important in apoptotic cell death by either opening a gateway for cytochrome *c* release from the mitochondria [74,202] or by activating other mitochondrial-related pro-apoptotic factors such as DIABLO/smac [72,195]. Inhibitors of the mPTP opening like cyclosporin A bind to cyclophilin D which is associated with the adenine nucleotide transporter (ANT), part of the multi protein complex of the mPTP [200], and modulate mitochondrial depolarization [229], cytochrome *c* translocation [230], and cell death [74,230]. On the contrary, mPTP openers such as the ANT-activator atractyloside trigger mPTP opening, cytochrome *c* release and apoptosis [229,230]. Interestingly, the mPTP possesses a benzodiazepine binding site and the binding of ligands such as PK11195 [36,55] and antagonists like flumazenil [69] have been shown to modulate the mPTP. Since flavonoids have been reported to bind to benzodiazepine binding sites of GABA-A and adenosine receptors [52,132], they might also exert an effect on the mPTP and, therefore, modulate cytochrome *c* release. In addition flavonoids have been found to bind to ATP-binding sites of proteins [43] such as the mitochondrial ATPase [53], calcium plasma membrane ATPase [10] protein kinase A [164], PKC [117] and topoisomerase [17] all of which must be considered in intracellular responses to oxidative insults. Thus, flavonoids might be able to modulate mitochondrial functions by binding to ATP binding sits on the ANT, ATPases or others. Indeed, bongkrekic acid an antagonist of the ATP-binding site of the ANT has been shown to prevent mPT and the early activation of JNK in MPP^+-induced neuronal injury as a model for Parkinson's disease [28].

Overall, flavonoids might influence the release of pro-apoptotic factors such as cytochrome *c* from the mitochondrion, thus providing an interesting hypothesis for future investigations.

A different interpretation of potential bioactive effects of flavonoids might be the upregulation of antioxidant enzymes such as SOD, catalase, glutathione peroxidase, and enzymes related to glutathione synthesis. Speculatively, this may occur by two means. Firstly, direct effects on the activity of transcription factors or signaling cascades leading to the transcription of the above enzymes. Secondly, some flavonoids or their oxidation products (phenoxyl radical, quinones) may induce moderate levels of oxidative stress based on their pro-oxidant effects which can induce the upregulation of antioxidant defence enzymes similar to the priming effect of low doses of radiation of low concentrations of hydrogen peroxide. The expression of some enzymes implicated in antioxidant defences such as the enzymes for glutathione synthesis [235] and heme oxygenase [37] exhibit ERK1/2 dependency in the regulation of their expression. Furthermore, the transcription of Cu/ZnSOD is regulated by the transcription factor ELK-1 [35] and the promoter for MnSOD expression contain binding sites for Sp1, AP-1, and CREB [35,47], all of which have been linked to regulation by ERK1/2 [34,182].

In addition nitric oxide has been identified as a potent modulator of mitochondrial function, as discussed before. Recent reports demonstrated that the induction of iNOS expression following treatment with LPS or interferon-γ [108], as well as the expression of nNOS requires the ERK1/2 signaling pathway, providing a potentially interesting link between MAPK signaling, nitric oxide production, and mitochondrial function. Since epicatechin and kaempferol have been shown to attenuate the oxidative stress-mediated activation of ERK1/2 [178] it remains to be established whether or not these flavonoids are capable of suppressing the production of $NO^{\bullet}$ in neurons and glial cells in vivo as reported for iNOS in cultured macrophages [99,109,154], and what influences this may have on cellular outcomes of oxidative insults.

Taken together the MAPK signaling cascade might exemplify a possible link between flavonoids and the modulation of cellular responses to oxidative stress. However, this link is circumstantial, speculative, and remains to be further investigated.

With respect to the overall subject concerning the potential use of flavonoids against neurodegeneration during aging or disease, the bioavailability of flavonoids especially, to the target tissues in the CNS, is of fundamental importance. The fact that flavonoids are extensively metabolised in gut and liver leads to questions about the conse-

quences of such metabolism with regard to their bioactivity. Glucuronidation, which increases the polarity of the compound, might decrease the accessibility of metabolites into cells and tissues. And indeed the data presented here indicate that glycosylated or glucuronidated flavonoids are much less effective compared to their corresponding aglycones. Thus, the loss in bioactivity might be based on the decline of cell accessibility. This is especially important with respect to the blood–brain barrier and entry to the brain. On the other hand, metabolism like *O*-methylation might increase access to cells and tissues since it increases the lipid solubility. Furthermore, the *O*-methylation of the catechol group in the B-ring of flavonoids will change their oxidizability and might, therefore, increase their stability.

Only a very few investigators have studied the influence of metabolism on the bioactivity of flavonoids [105,137,178,193,194], thus most of the effects reported based on in vitro experiments, cannot be extrapolated to in vivo situations. For example, one of the most intensively investigated flavonoids, quercetin, is often just used as the aglycone in cell culture systems and interesting biological activities such as modulation of the multidrug-resistance protein [118], induction of apoptosis [168], inhibition of the expression of nitric oxide synthase and cyclooxygenase [162], protection against oxidative stress [88] and modulation of the calcium homeostasis [209] have been reported. However, in plants and, therefore, also in all plant-derived foods and beverages, quercetin is always present in a glycosylated form which completely alters its bioactivity. If digested the glycosides might be taken up or are cleaved and the released quercetin is metabolised, so that there is almost no free quercetin aglycone in circulation. Thus, the bioactivities observed using the aglycone alone may be very different form the effects of the derivatives of quercetin. It is, therefore, vital for cell culture experiments to analyze, synthesize and investigate the in vivo metabolites of the flavonoid under investigation. Furthermore, the availability of flavonoids to the brain remains to be investigated further. But perhaps the beneficial effects on memory, motoric functions and against age-related cognitive decline reported so far are not exerted directly in the brain but occur in the periphery and reflect subsequently on to the functions of the CNS. It might be that factors like platelet aggregation, vascular tone, hormonal changes, and immune responses mediated by flavonoid intake induce changes in the CNS.

8. Summary

MAPK signal transduction pathways are important for the evaluation and translation of oxidative/nitrosative stimuli into specific cellular responses. The role of MAPK signaling in neurodegeneration, in particular with respect to oxidative/nitrosative stress-induced neuronal death, is gradually emerging, implicating the manipulation of these signal transduction pathways as a potential strategy for therapeutic interventions.

The precise mechanisms of the neuroprotection exerted by flavonoids remain to be established. However, it is becoming increasingly clear that the classical hydrogen-donating activity per se is unlikely to be the sole explanation for the biological properties of flavonoids. This is based on the following arguments: firstly, flavonoids are extensively metabolised in vivo resulting in a significant alteration of their hydrogen-donating properties as well as their lipophilicity, i.e. accessibility into tissues. Secondly, concentrations present in plasma and tissues as in the brain are lower than those of ascorbate and Vitamin E, compounds that are considered to be antioxidants in vivo. Thirdly, other biological activities of flavonoids/metabolites have been reported including binding to ATP- and benzodiazepam-binding sites and their effects on enzyme activity, components of intracellular signaling and gene expression. The ability of flavonoids to prevent neuronal cell death following oxidative insults might depend on modulation of intracellular signaling cascades, such as the MAPK signal transduction pathway, and their proposed effects on mitochondrial function. To substantiate further a role for flavonoids as neuroprotective agents, advances are needed especially with regard to understanding accessibility to the brain, the identification of precise molecular targets and the confirmation of the proposed role of flavonoids in the complex organization of intracellular signaling.

With regard to $NO^{\bullet}$, the other manipulator/mediator of cellular responses discussed in this review, the physical, chemical and biological properties of $NO^{\bullet}$ make this molecule a potent endogenous modulator of signal transduction. The cellular effects of $NO^{\bullet}$ depend on its intracellular concentration, the redox status of the cell, the presence of other ROS/RNS and on the dynamics of its generation. Interestingly, accumulating data suggest that the regulation of the expression of nitric oxide synthases depends, at least in part, on MAPK signaling, thereby offering a mechanism of cross-talk between components participating in redox-sensitive cellular functions.

Our overall aim was to elucidate the potential, differential effects of dietary flavonoids, on the one hand, and endogenous $NO^{\bullet}$, on the other hand, as modulators of neurodegeneration, especially with respect to cellular signaling. This should provide stimulating hypotheses for future investigations.

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