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Green tea catechins as brain-permeable, non toxic iron chelators to "iron out iron" from the brain

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Summary Evidence to link abnormal metal (iron, copper and zinc) metabolism and handling with Parkinson's and Alzheimer's diseases pathology has frequently been reported. The capacity of free iron to enhance and promote the generation of toxic reactive oxygen radicals has been discussed numerous times. Metal chelation has the potential to prevent iron-induced oxidative stress and aggregation of alpha-synuclein and beta-amyloid peptides. The efficacy of iron chelators depends on their ability to penetrate the subcellular compartments and cellular membranes where iron dependent free radicals are generated. Thus, natural, non-toxic, brain permeable neuroprotective drugs, are preferentially advocated for "ironing out iron" from those brain areas where it preferentially accumulates in neurodegenerative diseases. This review will discuss the most recent findings from *in vivo* and *in vitro* studies concerning the transitional metal (iron and copper) chelating property of green tea and its major polyphenol, (−)-epigallocatechin-3-gallate with respect to their potential for the treatment of neurodegenerative diseases.

Abbreviations: *AD* Alzheimer's disease; *Aβ* amyloid beta peptide; *APP* amyloid precursor protein; *DA* dopamine; *DFO* desferrioxamine; *EGCG* (−)-epigallocatechin-3-gallate; *HIF-1* hypoxia inducible factor-1; *IRE* iron responsive element; *IRP* iron regulatory protein; *MPTP* N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; *6-OHDA* 6-hydroxydopamine; *OS* oxidative stress; *PD* Parkinson's disease; *PKC* protein kinase C; *sAPP-α* soluble APP-alpha; *SN* substantia nigra; *Tf* transferrin; *TfR* transferrin receptor; *ROS* reactive oxygen species.

Introduction

The consumption of tea is believed to have been initiated five thousands years ago in China and India (Gutman, 1996). In general, tea is consumed in the form of green tea, oolong tea or black tea, which are all derived from *Camellia sinensis*, a small plant grown mainly in China, Japan and Southeast Asia. Green and black teas are differently manufactured. Preservation of the intact green leaf is of highest importance in the preparation of green tea. The freshly harvested leaves are steamed to prevent fermentation, rolled and then dried. This process yields a chemical composition in green tea similar to the fresh tea leaf. The preparation of black tea involves a "fermentation" process in which fresh leaves are withered, rolled and crushed, initiating a chain of oxidative reactions of the catechin polyphenols contained in them. This results in polymerization of the catechins converting them into higher molecular weight theaflavins and thearubigins, conferring tea its strong dark color and special flavor. A less extensive, partial fermentation leads to a lighter flavored tea, known as oolong tea (Cooper et al., 2005).

Nowadays, tea is considered as a source of dietary constituents endowed with biological and pharmacological activities with potential benefits to human health. The increasing interest in the health properties of tea extract and its main catechin polyphenols have led to a significant rise in scientific investigation for prevention and therapeutics in several diseases. Several of these are subject, in the last few years, of intensive investigation in diverse medical disciplines, such as cardiology, oncology (Wiseman et al., 2001; Higdon and Frei, 2003; Galati and O'Brien, 2004), inflammatory diseases and neurology. The favorable properties of green tea extract had been ascribed to their high content of polyphenolic flavonoids. Fresh tea leaves, contains a high amount of catechins, a group of flavonoids or flavanols, known to constitute 30–45% of the solid green tea extract (Yang and Wang, 1993; Wang et al., 1994). Among the tea catechins, (−)-epigallocatechin-3-gallate (EGCG) is the major constituent, accounting for more than 10% of the extract dry weight followed by (−)-epigallocatechin, (EGC) > (−)-epicatechin and (EC) ≥ (−)-epicatechin-3-gallate (ECG). Ingested tea catechins are absorbed mainly in the small intestine and metabolized by enzymatic reactions of glucuronidation, sulfation and *O*-methylation.

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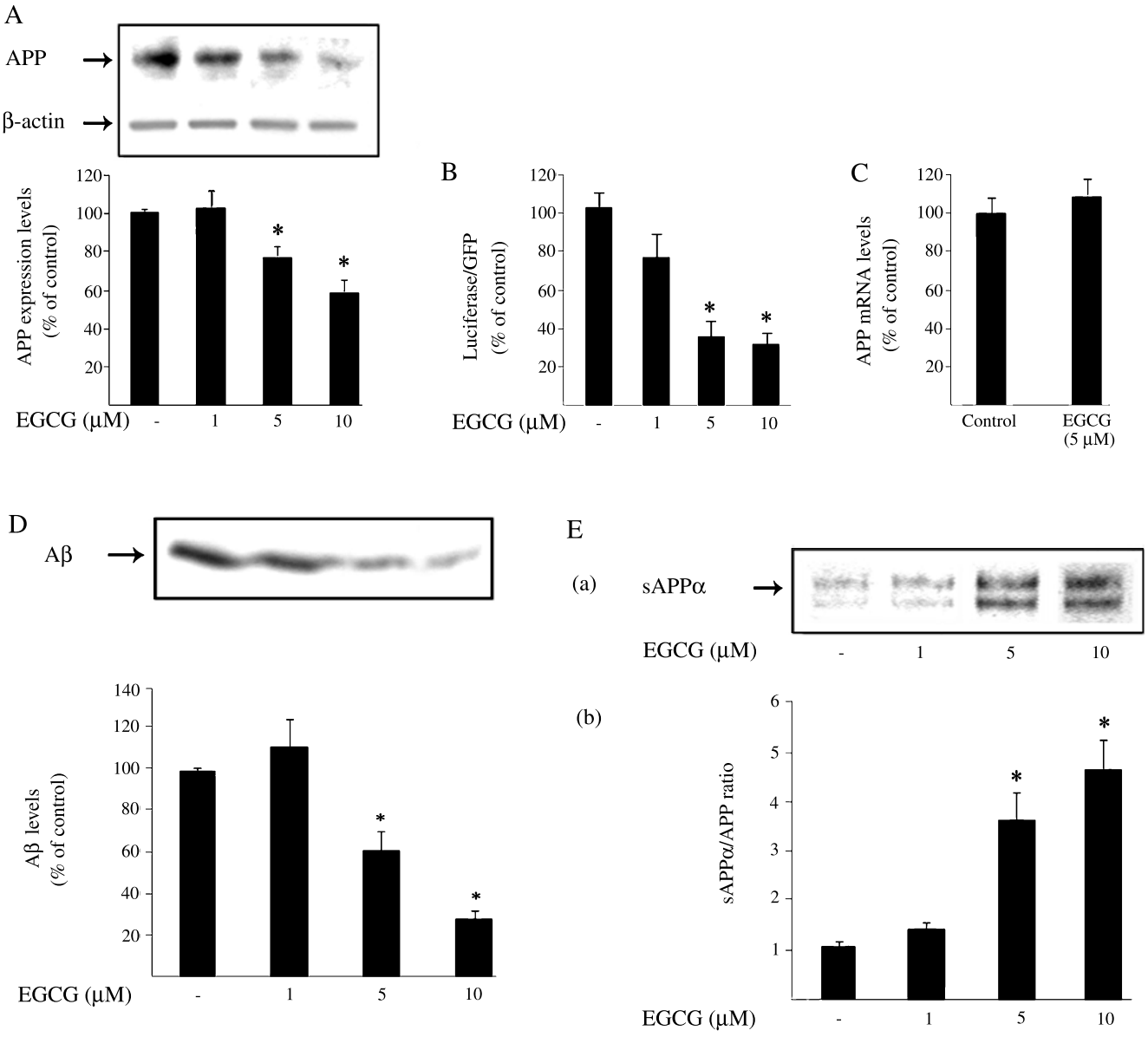

Fig. 1. Effect of EGCG on holo-APP protein and mRNA expression levels, sAPPα secretion and Aβ generation (adapted from Reznichenko et al., 2006). **A**, **B** Human neuroblastoma SH-SY5Y cells were treated without (control) or with increasing concentrations of EGCG (1–10 μM) for 48 h. **A** EGCG dose-dependently reduced holo-APP protein levels (43.1 ± 5.7% at 10 μM), as assessed by Western blot analysis, using antibody 22C11. The histogram under the gel micrograph summarizes the values from three independent experiments normalized to β-actin and expressed as percentage of control. $^{*}p < 0.01$ vs control. **B** The efficacy of EGCG as an iron chelator, to modulate the translation of a luciferase reporter gene driven by the APP 5′-UTR sequences was tested in U-87-MG glioma cells, co-transfected with 10 μg of DNA from pGALA plasmid (APP 5′-UTR + APP 3′-UTR sequences) and 5 μg of DNA from a construct that expresses GFP, to standardize for transfection efficiency. Cell plates were grown in the absence (control) or presence of increasing concentrations of EGCG (1–10 μM) for 48 h. Values represent luciferase activity normalized to GFP (mean ± SEM, from four independent experiments, each conducted in six replicates). $^{*}p < 0.01$, vs untreated control. EGCG gradually suppressed APP 5′-UTR reporter gene expression in a concentration-dependent manner (maximal inhibition of 68.4 ± 5.0% at 10 μM EGCG vs. the untreated control). **C** APP gene expression was measured by quantitative real-time RT-PCR. The amount of the products was normalized to the housekeeping gene 18S-rRNA and expressed as percentage of control. EGCG did not affect APP mRNA levels, indicating a post-transcriptional regulatory mechanism. **D** The regulatory effect of EGCG on the amyloidogenic Aβ peptides was analyzed in CHO/ΔNL cells, stably transfected with the ''Swedish'' APP mutation, since Aβ levels in the medium of SH-SY5Y are undetectable. EGCG treatment for 48 h markedly reduced Aβ levels in the medium of CHO/ΔNL cells, achieving a maximal decrease (72.8 ± 4.7%) with 10 μM EGCG. **E** The effect of EGCG on secreted soluble APP-alpha. sAPPα was assessed in SH-SY5Y cells under long-term, 48 h culture conditions. (a) EGCG significantly induced sAPPα release into the medium (at 10 μM, 241 ± 19% of control) and (b) progressively increased the ratio sAPPα/holo-APP along the EGCG concentration range (at 10 μM, 460% of control), indicating that the compound favors the non-amyloidogenic pathway of APP processing

These forms are detected in plasma and excreted in bile and urine (for review see Bravo, 1998). There is evidence that polyphenol metabolites and their parent compounds have access to the brain. Studies with radioactively labeled EGCG in mouse or chemiluminescence-based detection of EGCG in rats, demonstrated its incorporation into brain, as well as in various organs including kidney, heart, liver, spleen and pancreas (Nakagawa and Miyazawa, 1997; Suganuma et al., 1998).

Catechin polyphenols have been demonstrated to act directly as radical scavengers of oxygen and nitrogen species and exert indirect antioxidant effects through activation of transcription factors and antioxidant enzymes such as catalase and superoxide dismutase, thus modulating the cellular redox state (see reviews: Wiseman et al., 1997; Rice-Evans, 2001; Higdon and Frei, 2003). In addition to their radical scavenging action, green tea catechins possess well established metal chelating properties. Structurally important features defining their chelating potential are the 3′,4′-dihydroxyl group in the B ring (Hider et al., 2001), as well as the gallate group (Guo et al., 1996; Kumamoto et al., 2001), which may neutralize ferric iron to form redox-inactive iron, thereby protecting cells against oxidative damage (Grinberg et al., 1997). In fact, the ability of green tea catechins to act as antioxidants *in vitro* is based on their metal chelating capacity and on the potent quenching of singlet oxygen (Tournaire et al., 1993).

This review aims to shed light on the relevance of the divalent metal chelating properties of green tea polyphenols to neurodegeneration, where increased amounts of ionic, redox-active toxic metals trigger a cascade of neurotoxic events and cause aggregation of proteins such as alpha-synuclein (α-synuclein) and amyloid-β peptide (Aβ).

Iron and neurodegeneration

Various metals have been implicated in the pathophysiology of certain neuropsychiatric diseases. Thus, iron is present in substantia nigra, globus pallidus, and dentate gyrus at a concentration equal to or greater than that found in the liver. These three brain regions are known to be associated with neurodegenerative diseases (Youdim and Riederer, 2004). Specifically, redox-active iron has been observed in the rim of Lewy body, the morphological hallmark of Parkinson's disease (PD), composed also of lipids, aggregated α-synuclein (concentrating in its peripheral halo) and ubiquitinated, hyperphosphorylated neurofilament proteins (Jellinger, 2003). Altered iron homeostasis has also been reported in Alzheimer's disease (AD), as indicated by changes in the levels of iron, ferritin and transferrin receptor (TfR) in the hippocampus and cerebral cortex (Beard et al., 1993; Sipe et al., 2002; Honda et al., 2005). Iron promotes both deposition of Aβ and induction of oxidative stress (OS), which is associated with the plaques. Indeed, it has been demonstrated that amyloid deposits are enriched with zinc, iron and copper (Atwood et al., 2003).

Conventional neurochemical studies as well as genomic and proteomic profiling of brain autopsy material from PD patients and more recently from AD, have provided evidence for the involvement of supplementary processes, including glutamatergic neurotoxicity, nitric oxide elevation, dysfunction of ubiquitin-proteasome system and mitochondria, which may lead to breakdown of energy metabolism and consecutive intraneuronal calcium overload, increased expression of apoptotic proteins and loss of tissue reduced glutathione (GSH; an essential factor for removal of hydrogen peroxide) (Riederer et al., 1989; Blum et al., 2001; Linazasoro, 2002; McNaught et al., 2002; Blalock et al., 2004; Grunblatt et al., 2004; Poon et al., 2005; Zhang et al., 2005). These series of neurotoxic events may act independently or cooperatively, leading eventually to the demise of the neurons. Thus, considering the multifactorial nature of neurodegenerative disorders, drugs directed against single functional components of the different disease pathologies, such as cognition or movement disorder will be limited in efficacy. It is likely that strategies considering the application of multi-site directed drugs (polypharmacology) or combining drugs with different therapeutic targets may be more suitable to address the varied pathological aspects of the disease.

Neuroprotection by metal chelation with EGCG

One innovative therapeutic approach could be the use of non-toxic, brain-permeable natural plant polyphenols, reported to possess multifunctional activities (Guo et al., 1996; Morel et al., 1999; Hider et al., 2001; Rice-Evans, 2001; Joseph et al., 2005) and as recently reviewed (Mandel et al., 2004b, 2005). Of particular importance to neurodegenerative diseases, such as PD, AD and amyotrophic lateral sclerosis (ALS), is the ability of catechins to act as antioxidants, to inhibit peroxynitrite-mediated oxidation of dopamine (DA), to inhibit nitration of tyrosine residues (Pannala et al., 1998; Kerry and Rice-Evans, 1999) and to chelate divalent metals (Guo et al., 1996; Kumamoto et al., 2001). Research from our laboratory has demonstrated that the antioxidant-iron chelating activity of the major green tea polyphenol EGCG plays a major role in the prevention of neurodegeneration in a variety of cellular and animal models of neurodegenerative diseases (Mandel

et al., 2004a; Mandel and Youdim, 2004). Thus, EGCG was reported to protect human neuroblastoma cells from damage induced by 6-hydroxydopamine (6-OHDA) and 1-methyl-4-phenylpyridinium (MPP^+) (Levites et al., 2002); to protect primary hippocampal neurons (Choi et al., 2001) and rescue rat pheochromocytoma (PC12) cells from Aβ-induced toxicity, presumably through the scavenging of ROS (Levites et al., 2003). More recently, EGCG was shown to exert a neurorescue activity in long-term serum-deprived PC12 cells and to promote neurite outgrowth (Reznichenko et al., 2005). This could have important implications with regard to aging, PD and AD, suggesting a potential therapeutic use of EGCG in regenerating injured neuronal cells.

Accumulating studies indicate that the neuroprotective/neurorescue activity of catechin flavonoids are likely the result of activation of an array of different signaling pathways involved in cellular survival, growth and differentiation as protein kinase C (PKC) and extracellular mitogen-activated protein kinase (MAPK) (Schroeter et al., 2002; Mandel et al., 2005). In the context of cell survival, EGCG was shown to down-regulate pro-apoptotic genes, such as bad, bax, mdm2, caspase-1, cyclin dependent kinase inhibitor p21 and TNF-related apoptosis-inducing ligand (TRAIL) (Levites et al., 2002; Weinreb et al., 2003) and to regulate transcriptional activation (Wiseman et al., 1997; Higdon and Frei, 2003; Townsend et al., 2004; Zhou et al., 2004; Thomas and Kim, 2005). These findings and the well acknowledged antioxidant/iron chelating attributes of tea catechins, suggest that green tea extract may be a source of neuroprotectants, with particular relevance to neurodegenerative diseases where OS has also been implicated.

Reduction of amyloid precursor protein (APP) and toxic Aβ by EGCG: implication of iron chelation

Whether iron has a primary or a secondary role in neurodegeneration is unknown. The limited number of neuroprotective studies that have been carried out so far, indicate that iron-chelation therapy could be a viable neuroprotective approach for neurodegenerative disorders (Rogers and Lahiri, 2004; Zecca et al., 2004; Youdim and Buccafusco, 2005). Treatment with desferal/desferrioxamine (DFO) as an iron chelator or with the antibiotic iron and copper chelator, 5-chloro-7-iodo-8-hydroxyquinoline (clioquinol) was shown to be neuroprotective against the neurotoxins 6-hydroxydopamine (6-OHDA) and N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced neurotoxicity in rats and mice, respectively (Ben-Shachar et al., 1991; Kaur et al., 2003). Furthermore, treatment with clioquinol inhibited Aβ accumulation in AD transgenic mouse model (Cherny et al., 2001). However, DFO is a very poor brain penetrating agent and clioquinol is highly toxic (Meade, 1975). More recently, the multifunctional iron chelator/monoamine oxidase (MAO)-A and -B inhibitor, brain-permeable compound, M-30 (Youdim et al., 2004; Youdim and Buccafusco, 2005), showed neuroprotective activities in neuronal rat PC12 and P19 cell cultures against serum deprivation and 6-OHDA (Zheng et al., 2005a, b) and in MPTP-induced parkinsonism (Gal et al., 2005).

In AD pathology, iron may regulate APP translation, via a mechanism involving the iron responsive element-type II (IRE-type II) located in the 5′UTR region of APP mRNA (Rogers et al., 2002). This is consistent with biochemical evidence pointing to APP as a redox-active metalloprotein (Huang et al., 2004). APP was found to be post-transcriptionally regulated by iron regulatory proteins (IRPs), which are labile iron pool-sensitive cytosolic RNA proteins binding specifically to the IRE located in the 5′ or 3′ untranslated regions of iron metabolism-associated mRNAs. Changes in the iron status (iron overload or depletion) lead to compensating changes in the IRP/IRE system of translational control of iron homeostasis. For example, the APP 5′-UTR-conferred translation was selectively down-regulated upon intracellular iron chelation, in a similar manner as the iron-storage protein ferritin, which also possesses an IRE in its 5′UTR mRNA (Rogers et al., 2002).

The evidence that tea catechins, including EGCG, are well established metal ion chelators and the involvement of metal chelation in APP/Aβ regulation raised the question whether EGCG could affect APP processing. This possibility was recently investigated by our group, demonstrating a dual regulatory effect of EGCG on Alzheimer's APP: EGCG reduces holo-APP, presumably through iron chelation, and induces non-amyloidogenic soluble APP-alpha (sAPPα) release via PKC activation, resulting ultimately in inhibition of Aβ peptide generation (Reznichenko et al., 2006) (Fig. 1A). The observed reduction in APP protein levels was linked to the chelation of intracellular free-iron labile pool by EGCG, as this polyphenol was shown to suppress the translation of a luciferase reporter gene driven by the APP 5′-UTR sequences (Fig. 1B), as has been recently described for other metal chelators, such as DFO, clioquinol and dimercaptopropanol (Rogers et al., 2002; Payton et al., 2003). Since EGCG did not alter APP mRNA levels (Fig. 1C), the decrease in the levels of APP protein was attributed to the suppression of APP translation. The finding that exogenous iron supplementation

reversed EGCG action on APP, reinforces the likelihood that these effects are mediated through modulation of the intracellular iron pool. The *in vitro* findings are supported by a previous *in vivo* study, which demonstrated that prolonged administration of EGCG to mice induced a significant reduction in membrane-associated APP levels in mice hippocampus (Levites et al., 2003). Furthermore, different experimental strategies have shown that EGCG markedly reduced secreted Aβ levels in the conditioned medium of Chinese hamster ovarian cells, overexpressing ''Swedish'' mutated APP (CHO/ΔNL) (Reznichenko et al., 2006) (Fig. 1D) and in primary neuronal cells derived from transgenic mice bearing the APP ''Swedish'' mutation (Rezai-Zadeh et al., 2005). Other lines of research focusing on Aβ stability and toxicity reported that green tea or wine polyphenols (e.g. resveratrol) are able to inhibit formation, extension and destabilization of Aβ fibrils *in vitro* (Ono et al., 2003, 2004), to lower the levels of secreted and intracellular Aβ in various cell lines (Marambaud et al., 2005) and to protect against Aβ-induced neurotoxicity (Levites et al., 2003). Thus, attenuation of both APP synthesis and Aβ production by EGCG could be of therapeutic value for AD therapy, as increased generation of β-amyloid peptides plays a central role in AD plaque formation (Cuajungco et al., 2005).

The other important pharmacological action of EGCG is related to recent reports demonstrating that either short- or long-term incubation with EGCG promotes the generation of the soluble N-terminal fragment, sAPPα, via PKC-dependent activation of α-secretase (Reznichenko et al., 2006; Levites et al., 2003) (Fig. 1E). In this context, EGCG has been shown to up-regulate PKCα and PKCε isoforms in mice striatum and hippocampus (Levites et al., 2003; Mandel et al., 2004a). New supportive data came from a study conducted in Alzheimer transgenic mice, showing that EGCG promotes sAPPα generation through activation of α-secretase cleavage (Rezai-Zadeh et al., 2005). This was accompanied by a significant reduction in cerebral Aβ levels and β-amyloid plaques. Since sAPPα and Aβ are formed by two mutually exclusive mechanisms, stimulation of the secretory processing of sAPPα might prevent the formation of the amyloidogenic Aβ. Thus, EGCG may influence Aβ levels, either via translational inhibition of APP or by stimulating sAPPα secretion. Cleavage of APP within the Aβ domain by α-secretases is of physiological interest, not only because it precludes the formation of Aβ, but also because it promotes the generation of sAPPα that exhibits neuroprotective properties (Mattson, 1997; De Strooper and Annaert, 2000). Moreover, shedding of the ectodomain is a prerequisite for the cleavage of the intracellular domain of γ-secretases, a process that liberates a C-terminal fragment with transcriptional activity (Cao and Sudhof, 2001; Gao and Pimplikar, 2001; Leissring et al., 2002). Thus, promotion of α-secretase-mediated APP processing, rather than down-regulation of Aβ production, may offer a novel approach to AD treatment (Esler and Wolfe, 2001).

Induction of iron/hypoxia-responsive genes by green tea catechins

The interplay of iron and oxygen is most interesting in hypoxic condition where they interact causing brain damage. The link between hypoxia and iron is reflected by the hypoxic-mediated regulation of proteins that modulate iron homeostasis like IRPs (IRP1 and IRP2), ferritin and TfR. Indeed, all the major genes of iron metabolism respond to hypoxia (Sorond and Ratan, 2000). Substantial amounts of chelatable iron are released from storage during hypoxia and ischemia (Bralet et al., 1992). One of the adaptive responses employed by hypoxic mammalian cells is the induction of hypoxia-inducible factor-1 (HIF-1), considered as the master regulator of the ''hypoxic world'', being responsible for the concerted expression of a myriad of genes which participate in the processes of angiogenesis, cell proliferation/survival and glucose/iron metabolism (Lee et al., 2004; Sharp and Bernaudin, 2004). Thus, the reduction in the small, chelatable iron pool by iron chelation will affect not only the post-transcriptional regulation of iron homeostasis-related mRNAs (e.g. TfR, ferritin) but also the induction of a wide array of genes tightly regulated by HIF-1 (Fig. 2).

Iron was recently shown to block HIF-1 activation induced by the green tea catechins, EGCG and epicatechin-3-gallate (ECG), as well as by DFO (Zhou et al., 2004; Thomas and Kim, 2005). In fact, both HIF-1 and IRP2 share a common iron-dependent proteasomal degradation pathway, by the activation of key iron and oxygen sensors prolyl hydroxylases, which become inactivated by iron chelation (Hanson et al., 2003; Wang et al., 2004). Thus, the reduction in the free-iron pool by EGCG chelation may result in the inhibition of prolyl hydroxylases and consequently, in the concerted activation of both HIF and IRP2. As IRPs and HIF-1 coordinate the expression of a wide array of regulators involved in cellular iron homeostasis, survival and proliferation (Templeton and Liu, 2003; Sharp and Bernaudin, 2004), their activation could be of major importance in neurodegenerative diseases (Fig. 2).

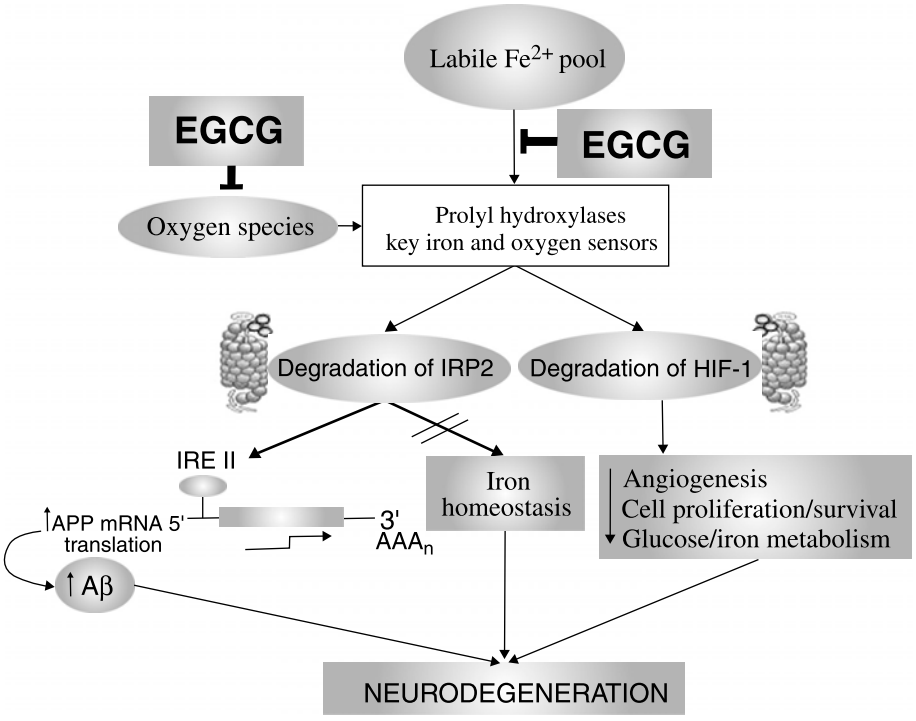

Fig. 2. Iron-induced neurodegeneration in AD via transcriptional activation of APP mRNA and suppression of hypoxia-inducible genes. Increase in labile Fe^{2+} pool can elevate the production of APP via proteasomal-mediated inactivation of IRP2, thereby promoting the translation of APP mRNA from its 5′UTR-typeII). Increased iron and oxygen species may activate the prolyl hydroxylase enzymes, which are key iron and oxygen sensors, leading to proteasomal-mediated degradation of the transcription factor HIF-1, a master regulator orchestrating the coordinated induction of a wide array of survival genes. It has been suggested that IRP2, similar to HIF-1, can be enzymatically modified by a prolyl hydroxylase, routing it to proteasomal degradation. Both iron chelation and oxygen species scavenging by EGCG may prevent the degradation of IRP2 and HIF-1, resulting in the promotion of cell survival processes such as angiogenesis, glucose metabolism and maintenance of iron homeostasis. EGCG, (−)-epigallocatechin-3-gallate; IRP, iron regulatory protein; HIF-1, hypoxia inducible factor-1. Sharp arrows indicate positive inputs, whereas blunt arrows are for inhibitory inputs. For a more detailed explanation read text

Conclusion

Although the precise mechanism of neuroprotection/neurorescue exerted by green tea catechins is not fully established, accumulating evidence indicates the participation of multiple pathways, including the pro-survival PKC and extracellular mitogen-activated protein kinase (MAPK) signaling (Mandel et al., 2005); promotion of neurite outgrowth (Reznichenko et al., 2005); down-regulation of pro-apoptotic genes (Levites et al., 2002; Weinreb et al., 2003) and promotion of secreted soluble, non-toxic, non-amyloidogenic form of APP, reputed to have neurotrophic and neuroprotective properties against excitotoxic and oxidative insults (Levites et al., 2003). Recently, a new dimension was added to these actions, associated with the iron chelating property of green tea catechins and the impact on neurodegenerative processes, as inhibitors of OS-mediated protein aggregation, APP synthesis and Aβ plaque formation. Considering the pathological role iron plays in a number of neurological conditions, the use of EGCG as a natural, non-toxic, lipophilic brain permeable neuroprotective drug, could offer potential therapeutic benefits for ''iron out iron'' from those brain areas where it preferentially accumulates (Youdim and Buccafusco, 2005).

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The authors of this review would like to honour Moussa Youdim for his outstanding and significant contribution to the field of neuroscience. He was among the pioneers who envisaged the pivotal role iron takes in the ''neurodegeneration arena''. His high intellect, together with his innovative research on Parkinson's disease and multifunctional drug design, place him among the handful of people who make one feel that the ''sky is the limit''.

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