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COMPREHENSIVE INVITED REVIEW

Neuroprotective Multifunctional Iron Chelators: From Redox-Sensitive Process to Novel Therapeutic Opportunities

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Abstract

Accumulating evidence suggests that many cytotoxic signals occurring in the neurodegenerative brain can initiate neuronal death processes, including oxidative stress, inflammation, and accumulation of iron at the sites of the neuronal deterioration. Neuroprotection by iron chelators has been widely recognized with respect to their ability to prevent hydroxyl radical formation in the Fenton reaction by sequestering redox-active iron. An additional neuroprotective mechanism of iron chelators is associated with their ability to upregulate or stabilize the transcriptional activator, hypoxia-inducible factor-1α (HIF-1α). HIF-1α stability within the cells is under the control of a class of iron-dependent and oxygen-sensor enzymes, HIF prolyl-4-hydroxylases (PHDs) that target HIF-1α for degradation. Thus, an emerging novel target for neuroprotection is associated with the HIF system to promote stabilization of HIF-1α and increase transcription of HIF-1–related survival genes, which have been reported to be regulated in patient's brains afflicted with diverse neurodegenerative diseases. In accordance, a new potential therapeutic strategy for neurodegenerative diseases is explored, by which iron chelators would inhibit PHDs, target the HIF-1–signaling pathway and ultimately activate HIF-1–dependent neuroprotective genes. This review discusses two interrelated approaches concerning therapy targets in neurodegeneration, sharing in common the implementation of iron chelation activity: antioxidation and HIF-1–pathway activation. *Antioxid. Redox Signal.* 13, 919–949.

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I. Introduction

The pathogenesis of a number of neurodegenerative disorders, such as Parkinson's disease (PD), Alzheimer's disease (AD), and amyotrophic lateral sclerosis (ALS), as well as the aging process, is associated with increased levels of mitochondria-derived free radicals and oxidative damage (87, 280). The mitochondrion is one of the main sources of endogenous reactive oxygen species (ROS), and its proteins signify targets for oxidative alteration and loss in function (105). ROS can be radicals, possessing unpaired electrons, such as superoxide anion and hydroxyl radical or nonradical molecules, such hydrogen peroxide (H_2O_2) (307). Even so, ROS may not create a real risk to the neurons if the toxic end products are detoxified and balanced against the amount of ROS generated (307). Under physiologic conditions, superoxide is converted to H_2O_2 by the enzyme superoxide dismutase (SOD) or by interaction with transition metals (86). H_2O_2 is, in turn, reduced to water by glutathione peroxidase (GP) or converted to oxygen and water by catalase (86). Superoxide and H_2O_2 are relatively unreactive toward biologic processes; however, H_2O_2 has been reported to induce cell death in neuronal cell cultures (335, 344) through the formation of reactive hydroxyl radicals, which are generated by the interaction of H_2O_2 with transitional metals, such as iron, in the Haber-Weiss reaction/Fenton reaction (222). Thus, high levels of reactive iron can increase oxidative stress (OS)-induced neuronal vulnerability of environmental or endogenous toxins (354). Iron accumulation, and OS are early events in many neurodegenerative diseases, such as PD and AD, proximal to the development of hallmark pathologies, contributing significantly to the pathogenic processes of these disorders (354). A wealth of data has drawn attention to the significance of both maintaining reducing conditions in cells and the fight against the damaging effect of iron/ROS intermediates. Defense mechanisms include the reduction of ROS by iron chelators and radical scavengers and the induction of various antioxidant enzymes, such as catalase, manganese-, copper- and zinc-containing SOD (Mn-SOD/Cu-SOD/Zn-SOD), and the redox-sensitive enzyme GP (280) (Fig. 1).

Additionally, growing evidence supports the concept of oxygen signaling across membranes of intracellular com-

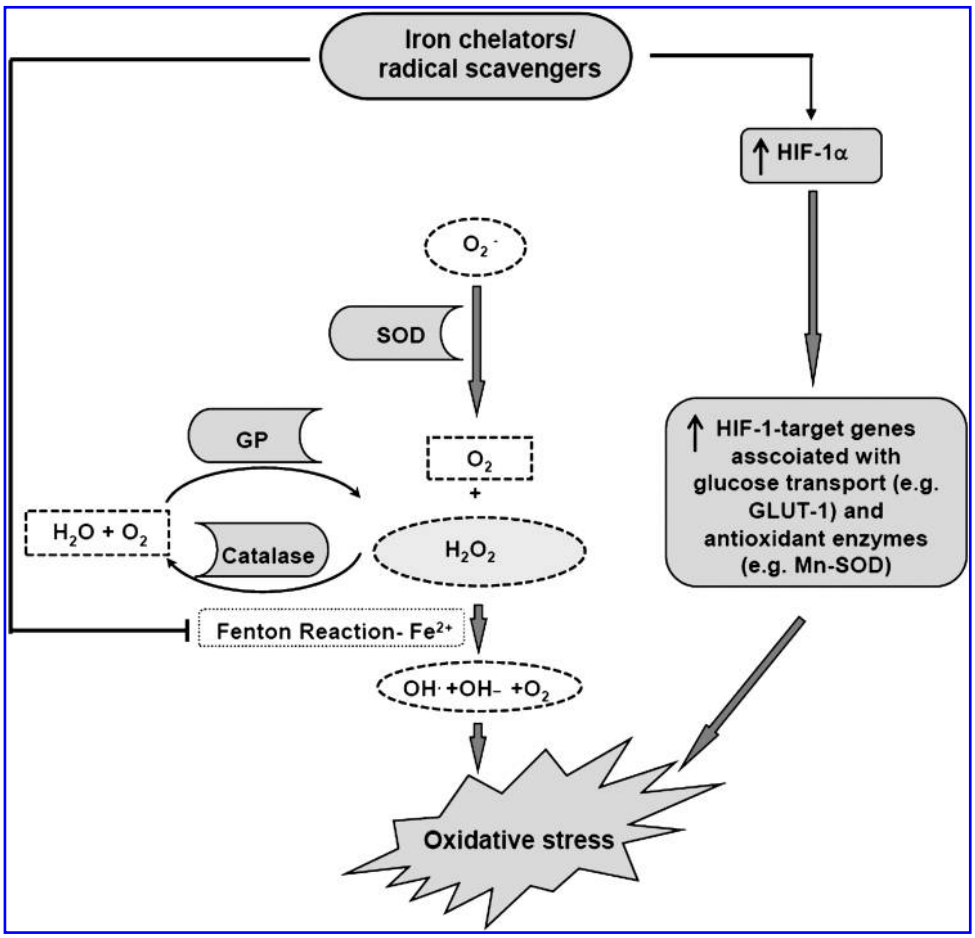

FIG. 1. Schematic diagram illustrating the potential neuroprotective effects of iron chelators and radical scavengers on attenuation of oxidative stress (OS). Under normal physiologic conditions, the use of oxygen by cells with aerobic metabolism generates potentially deleterious ROS, including superoxide (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^{\cdot}$). Superoxide is converted to H_2O_2 by the enzyme superoxide dismutase (SOD) or by interaction with transition metals. H_2O_2 is, in turn, reduced to water by glutathione peroxidase (GP) or converted to oxygen and water by catalase. Iron chelators/radical scavengers can inhibit the Fenton reaction and, alternatively, the induction of HIF-1α can affect glucose transport and enhance antioxidant enzymes (such as GLUT-1 and Mn-SOD, respectively), resulting in decreased OS.

partments, linked to a certain redox state and toxic harmful ROS, that might be crucial in regulating the level and pattern of gene expression of oxygen- and redox-responsive transcription factors (286). In the brain, such redox-sensitive transcription factors are suggested to be differentially regulated by oxygen availability, bind specific DNA consensus sequences, and activate the expression of several genes, particularly those controlling adaptive cellular homeostasis, known to compensate for OS (26, 282). Among such factors, the hypoxia-inducible factor (HIF)-1, whose activation states are differentially regulated under hypoxia, is particularly important (26, 282, 327, 352) (Fig. 2). In normoxia, HIF-1α is constrictively synthesized and immediately degraded by the ubiquitin-proteasome pathway mediated by specific prolyl residues that are hydroxylated by an enzyme family of HIF-prolyl-4-hydroxylases (HIF-PHD), requiring di-oxygen, 2-oxoglutarate and iron (Fe^{2+}) as co-substrates (279, 305).

PHD isoforms are known for regulating transcriptional regulators involved in hypoxic adaptation, such as HIF-1α and cAMP response element–binding protein (CREB) (292). Inhibition of PHDs enzymes under hypoxic or iron-chelating conditions results in heterodimerization and activation of nuclear translocation of HIF-1α protein and subsequently enhanced binding to hypoxia-responsive elements (HREs) of promoters of specific HIF-1–target genes that encode proteins involved in various adaptive/survival responses (116, 291). These include adrenomedullin (ADM), aldolase (ALD) A and C, enolase 1, erythropoietin (EPO), glucose transporter (GLUT) 1 and 3, hexokinase 1 and 2, $p21^{waf\ 1/cip1}$, transferrin, transferrin receptor (TfR), vascular endothelial growth factor (VEGF), and many others (282) that are involved in oxygen, iron, and energy homeostasis, prevention of ROS generation, and induction of neuronal outgrowth processes, leading to cell survival (77) (Figs. 1 and 2). Indeed, recent studies described small-molecule inhibitors of PHDs as a route to HIF-1 activation and neuroprotection [see review (112)]. It should be noted that HIF-1 activation may mediate prosurvival or prodeath responses, depending on the neuronal cell type examined and types of the insults (5).

Although the most-studied mechanism of HIF-1 induction is hypoxia, a variety of additional reagents and conditions result in stabilization of HIF-1 and induction of HIF-1–regulated genes, such as cobalt chloride ($CoCl_2$), interleukin-1β, tumor necrosis factor-α (TNF-α), transforming growth factor-β (TGF-β), insulin, insulin-like growth factor (IGF), heregulin, vitamin E, and the flavonoid quercetin (240, 287, 342, 356). In addition, an important class of HIF-1 inducers with direct relevance to neurodegenerative diseases includes the iron-chelating compounds (159, 168, 292). Thus, HIF-1α stabilization may be mediated by a decrease in ferrous ion (Fe^{2+}, located at the catalytic site of PHD), which is required by the enzyme to hydroxylate HIF-1α and target this transcription factor for ubiquitinylation and proteasomal degradation by the enzyme von Hippel-Lindau (pVHL) (110). It

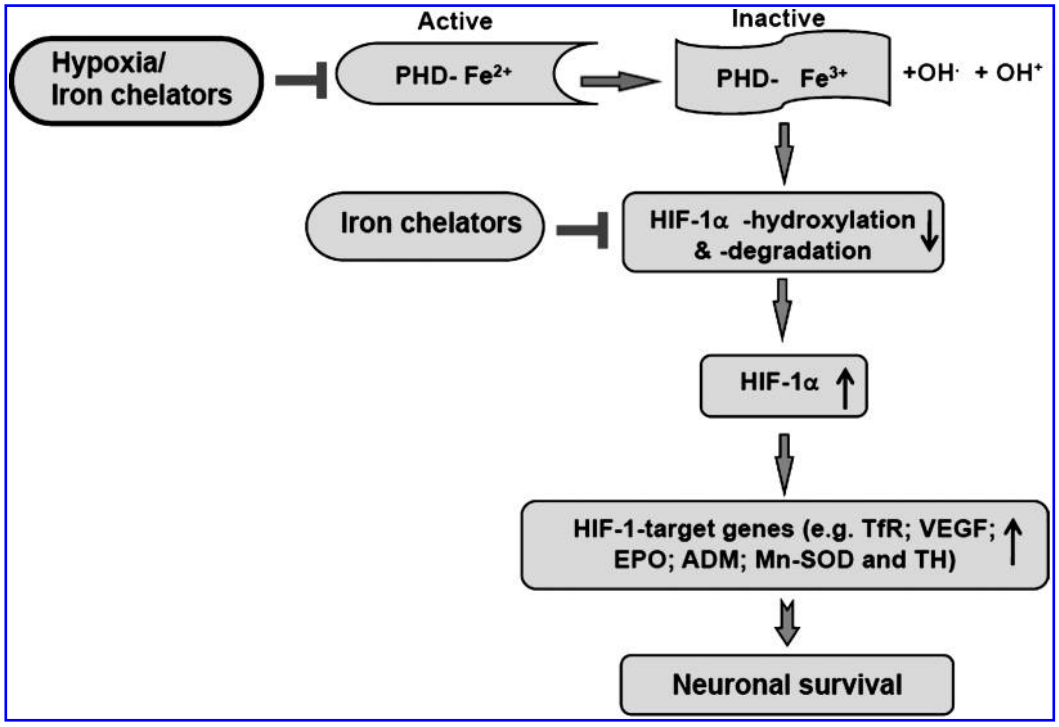

FIG. 2. Regulation of HIF-1α and neuronal survival under hypoxia and iron-chelation conditions. HIF-1α is degraded rapidly by the ubiquitin-proteasome pathway mediated by specific prolyl residues that are hydroxylated by an enzyme family of HIF/PHD, which are nonheme, iron (Fe^{2+})-dependent enzymes. Under hypoxic and iron-chelation conditions, hydroxylation is suppressed, allowing nuclear accumulation of HIF-α. Reduced activity of the PHDs initiates the stabilization of HIF-1α, heterodimerization and activation of nuclear translocation, and binding to hypoxia-response elements of promoters of specific HIF-1– adaptive target genes, such as glycolytic enzymes (*e.g.*, enolase 1, aldolase A, lactate dehydrogenase A), vascular endothelial growth factor (VEGF), erythropoietin (EPO), adrenomedullin (ADM), Mn-SOD, tyrosine hydroxylase (TH), and many others that are involved in the modulation of oxygen, iron, and energy supply, promoting neuronal survival processes.

is now likely that complexing the excess of Fe^{2+} ions with iron-chelating/radical scavenging compounds may favorably affect neuronal survival by a dual action, targeting ROS generation and HIF-1α induction (290, 293) (Figs. 1 and 2).

This review focuses on the regulatory effects of iron-chelating/radical scavenging agents regarding antioxidative activity and HIF-1α signaling neuroprotective induction, allied with modulation of HIF-1–target genes, as novel therapeutic strategies for neurodegenerative pathologies. In particular, we discuss the potential benefits of novel iron-binding, brain-permeable compounds, possessing multimodal activity with a special emphasis on the iron-modulated HIF-1α signal-transduction pathway.

II. Brain-Iron Homeostasis, Oxidative Stress, and Neurodegeneration

The etiology of neurodegenerative diseases is not yet well understood. However, over the last two decades, increasing evidence has suggested that progressive iron accumulation, iron-dependent OS, increased monoamine oxidase (MAO)-B activity (349, 354), as well as reduced antioxidant levels and activities in the brain, may be major pathogenic factors in neurodegenerative diseases (99, 104, 354).

In the central nervous system (CNS), iron is essential for multiple functions, including gene expression, DNA synthesis, neurotransmission, myelination, oxygen transport, storage and activation, mitochondrial electron transport, and many important metabolic processes (23, 106). Iron incorporation and transport in the brain is regulated by the interaction between the endothelial cells and astrocytes: the TfR1 in the luminal membrane of endothelial cells binds Fe^{3+}-loaded transferrin and internalizes this complex in the endosomal compartment, where Fe^{3+} is reduced to Fe^{2+}. The latter is transported across the endosomal membrane into the cytosol by the divalent metal transporter-1 (DMT1) and exported into the extracellular fluid by ferroprotein (265). Alternatively, it has been proposed that the transferrin-TfR1 receptor complex may be transported from the luminal to the abluminal surface, followed by iron release (209). Ceruloplasmin, expressed in the astrocyte, oxidizes newly released Fe^{2+} to Fe^{3+}, which binds to transferrin in the brain interstitial fluid (188, 209, 265). Fe^{2+} can also bind to ATP or citrate and be transported as nontransferrin-bound iron (NTBI), which is the source of iron for astrocytes and oligodendrocytes, which do not express TfR1 (209, 265).

Several lines of evidence indicate that iron accumulation and deposition in the brain can cause a vast range of neurodegenerative disorders (18, 24, 132, 297, 354, 355). High concentrations of reactive iron can induce OS, because of its interaction with H_2O_2 in the Fenton reaction, resulting in an increased formation of hydroxyl free radicals (297). Free radical–related OS causes molecular damage that can then lead to a critical failure of biologic functions, protein modification, misfolding and aggregation, and ultimately, cell death (105, 242, 243, 274) (Fig. 3). Additionally, iron accumulation might increase the neurotoxicity of endogenous or environmental toxins. Recently, iron homeostasis was linked to activation of the *N*-methyl-D-aspartic acid (NMDA) receptor; a signaling cascade that involves nitric oxide synthase (NOS) and adaptor proteins that interact with ferroprotein (49).

Iron accumulates in the brain as a function of age; this process involves the accumulation of iron-containing molecules (primarily ferritin) in the microglia, astrocytes, and

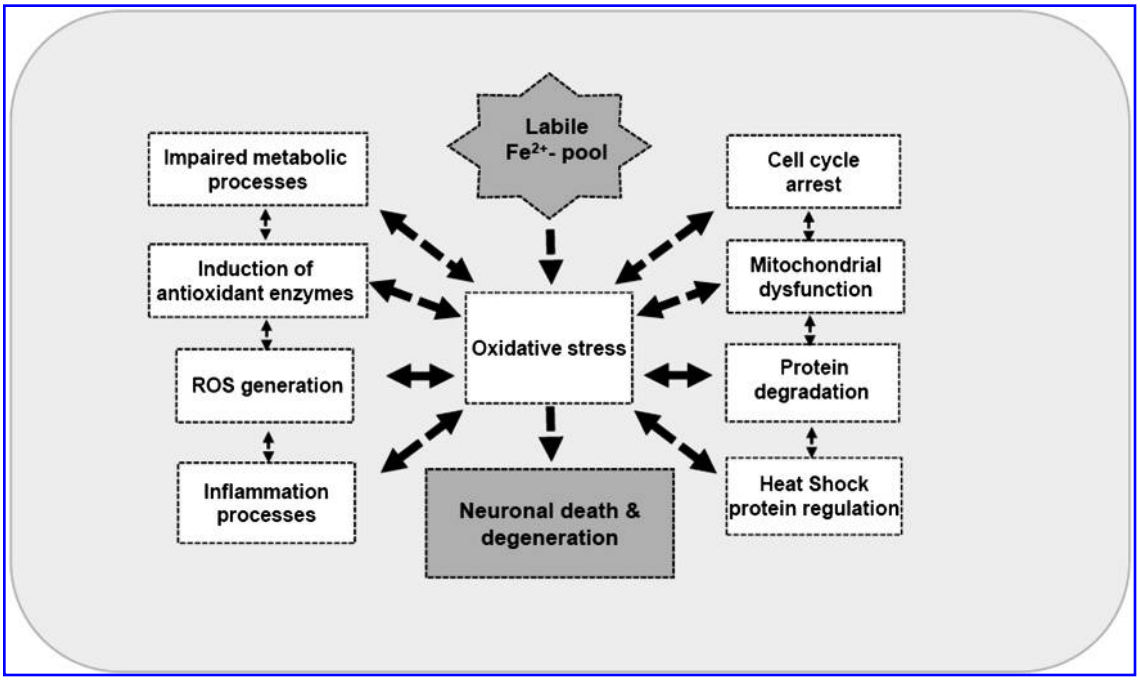

FIG. 3. Schematic illustration of the current hypothesis for labile Fe^{2+}-pool induced OS-neurodegeneration etiology. The cellular labile iron (Fe^{2+}) pool is a chelatable and redox-active iron that serves as a crossroad of cellular iron homeostasis and promotes the generation of ROS. The labile Fe^{2+} pool–induced OS has been suggested to cause a cascade of molecular damage that can lead to a critical failure of biologic functions, such as cell-cycle arrest, mitochondrial dysfunction, protein degradation, impaired metabolic processes, and inflammation processes, which ultimately result in neuronal death.

oligodendrocytes, particularly in the selective brain regions that are targeted in neurodegenerative diseases (*e.g.*, cortex, hippocampus, and substantia nigra (SN) (4, 18, 253, 348, 349, 354). This could result from alterations in brain vascularization that occur during aging and neurodegenerative diseases (37, 78, 302). Furthermore, during aging, brain iron is partially converted from its stable/soluble form, (ferritin), to a highly reactive form (hemosiderin) and other oxyhydroxides [see review (62)].

The following section discusses several neurodegenerative diseases with relevance to iron accumulation and OS and also addresses the potential of iron-chelating therapeutic strategies.

A. Parkinson's disease

PD is a neurodegenerative disease of the elderly, affecting 1% to 2% of those older than 60 years, causing motor dysfunctions, such as bradykinesia, resting tremor, rigidity, and postural instability, but also affecting autonomic functions and cognition (186, 226). Although this disease has long been considered a nongenetic disorder of "sporadic" origin, 5% to 10% of patients are now known to have monogenic forms (175). PD is characterized primarily by the progressive, selective, and irreversible loss of dopaminergic neurons in the SN pars compacta (SNpc) (50% to 70%) and subsequent decrease of dopamine (DA) concentrations in the striatum (120, 223). The neuropathologic hallmark of PD is the formation of eosinophilic Lewy bodies in surviving dopaminergic neurons (131). The nigral pathology in PD has been shown to be associated with OS, mitochondrial dysfunction, NOS toxic actions, excitotoxicity, inflammation, and defects in the ubiquitin-proteasome system (134, 136, 201). In addition, it has been shown that iron concentrations are significantly elevated in human parkinsonian SNpc within the melanized DA neurons (94, 98), as well as in the SNpc of 6-hydroxydopamine (6-OHDA) and *N*-methyl-4-phenyl-1,2,3,6-tertahydropyridine (MPTP) animal models of PD (95, 351). It is noteworthy that iron and ferritin accumulation occur within the neurons and oligodendrocytes in distinctive regions of the brain with ubiquitin-positive inclusion bodies (355). Targeted deletion of the gene encoding iron-regulatory protein 2 (IRP2) caused misregulation of iron metabolism and progressive neurodegeneration in mice, as evidenced by axonal degeneration in the CNS (165, 301) and movement disorder characterized by ataxia, bradykinesia, and tremor (165). Additional studies demonstrated that iron misregulation associated with the loss of IRP2 protein in the Ireb2(−/−) mice affected DA metabolism in the striatum, resulting from the loss of DA and DA-regulating proteins, thus supporting the view that the IRP2−/− genotype may enable neurobiologic events associated with PD and aging (165, 270).

As we have pointed out, iron participates in the Fenton chemistry, reacting with H_2O_2 to produce the most reactive ROS, the hydroxyl radical. The formation of the latter, combined with depletion of endogenous antioxidants, particularly tissue glutathione (GSH; the most common pathway of iron disposition in the brain), leads to OS (31, 109, 163). In the SN of parkinsonian brains, a drastic depletion occurs of endogenous antioxidants, such as reduced GSH (31, 133, 134, 237, 254). Iron also facilitates the decomposition of lipid peroxides to produce highly cytotoxic oxygen-related free radicals, which can cause damage to DNA, lipids, proteins, and ultimately, cell death associated with PD (Fig. 3). Previous evidence suggests that iron, together with H_2O_2, in the presence of DA, can induce the formation of the neurotoxic 6-OHDA (130, 182, 214). 6-OHDA may be also formed from L-dihydroxyphenylalanine (L-DOPA) in the presence of iron and hydrogen peroxide (190). Iron-dependent OS tends to be tissue specific, owing to differential neuronal cell susceptibility. This vulnerability is affected by three main parameters: (a) axonal length and thickness, (b) axonal sprouting, and (c) thickness of the myelin sheath (35).

Additional contribution to OS stems from elevated MAO-B levels, leading to increased DA production (135). This results in increased DA oxidation through both MAO activation and autooxidation, leading to elevated levels of H_2O_2, which in turn, participates in Fenton chemistry, thus creating a vicious circle (Fig. 4). Postmortem studies reported that MAO-B activity in the human brain is increased with age and in neurodegenerative diseases, in particular, PD (56, 81, 82). The increased activity level of MAO-B in the aged brain is thought to be associated with OS, which may play a role in the vulnerability of the DA system and age-related degeneration (82). These findings suggest that iron chelators, antioxidants, and MAO-B inhibitors may have great therapeutic potential for PD (21, 22, 91, 150, 357) (Fig. 4).

1. Therapeutic strategies in PD.

The knowledge that DA is equally oxidatively deaminated by MAO-A and -B and the dominance of MAO-B (80%), as compared with MAO-A in the extrapyramidal regions of the human brain, led to introduction of the irreversible selective MAO-B inhibitors, initially, selegiline [(*R*)-(-)-*N*,2-dimethyl-*N*-2-propynylphenethylamine]; L-deprenyl), and now, rasagiline [*N*-propargyl-1*R*(+)-aminoindan] (Fig. 5) as potential therapeutic drugs for PD (33, 174, 231, 232, 267, 304). Both compounds are approved by the United States Food and Drug Administration. Selegiline was the first in monotherapy (230) and as adjunct PD therapies in clinical neurology (33, 289, 306). A recent landmark report of a phase III delayed-start clinical trial in parkinsonian subjects (ADAGIO) indicated that rasagiline may be the first "disease-modifying" drug, with the benefits of slowing the disease beyond symptoms improvement (224, 225, 275).

Several studies have shown that both rasagiline and selegiline possess neuroprotective activities in various cell-culture and in *in vivo* preclinical models of PD (51, 73, 189, 334, 347, 362). It was suggested that MAO inhibition of rasagiline is not a prerequisite for its neuroprotective activity, as its *S*-isomer, TVP1022, which is more than 1,000 times less potent as a MAO inhibitor, exerted similar protective effects (345). We previously reported that the mechanisms whereby rasagiline exerts its multifactorial neuroprotective effects include upregulation of cellular antioxidant activity and antiapoptotic factors, associated with its propargyl moiety and 1-(R) aminoindan (15, 17, 334, 345). Interestingly, both rasagiline and selegiline exerted an antioxidant effect by enhancing the activities of enzymes (SOD and catalase) involved in OS in the dopaminergic system in the rat (43, 44, 153, 160, 194). In accordance, rasagiline was demonstrated to increase the expression levels of SOD and GP in serum- and nerve growth factor (NGF)- deprived pheochromocytoma (PC12) cells (312). Moreover, rasagiline was shown to upregulate the expression of various mitochondrial antiapoptotic members of Bcl-2

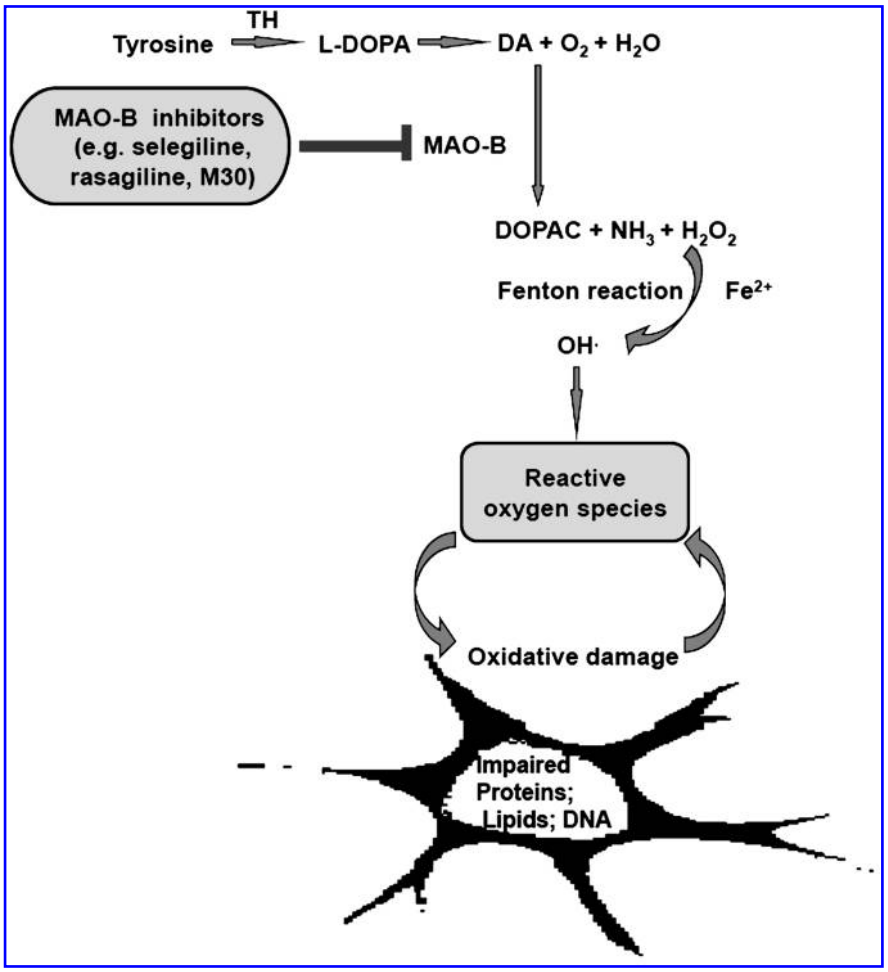

FIG. 4. Mechanism of MAO-B inhibitors–attenuated oxidative damage. DA oxidation, through MAO-B, leads to elevated levels of H_2O_2, which in turn, participates in Fenton chemistry, thus creating a vicious circle of oxidative damage. MAO-B inhibitors can inhibit generation of H_2O_2 and subsequently prevent neuronal damage.

family proteins (such as Bcl-2 and Bcl-xl), to prevent the mitochondrial permeability transition (PT), and to increase the levels of glial cell line–derived neurotrophic factor (GDNF) in human dopaminergic neuroblastoma SH-SY5Y cells (2, 194). These findings indicate the ability of rasagiline to adjust the apoptotic threshold and protect degenerating neurons in PD. More prominently, rasagiline has been found to possess an *in vivo* neurorestorative activity in SNpc neurons, when given after MPTP (268) or the proteasome inhibitor, lactacystin (362). This property has been suggested to be associated with activation of the neurotrophic tyrosine kinase receptor (Trk) pathway, involving stimulation of various cell signal-transduction pathways, such as protein kinase C (PKC) and Ras-phosphatidylinositol-3-kinase (PI3K)-Akt (268).

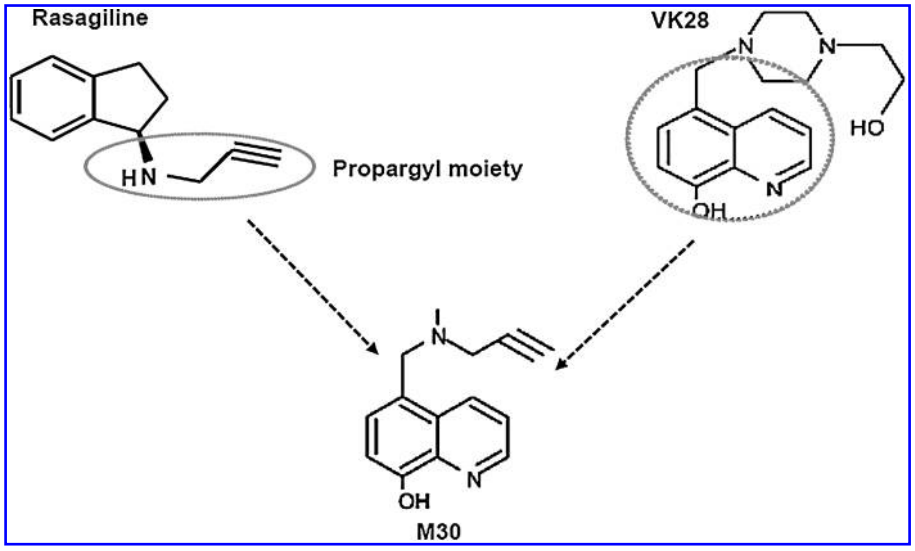

FIG. 5. Chemical structure of the brain-permeable multifunctional iron chelator/radical scavenger, M30 [5-(*N*-methyl-*N*-propargylaminomethyl)-8-hydroxyquinoline] and its parental compounds. This multimodal hybrid drug contains the propargyl moiety of the anti-parkinsonian MAO-B inhibitor drug, rasagiline and the antioxidant–iron chelating moiety of VK28 {5-[4-(2-hydroxyethyl) piperazine-1-ylmethyl]-quinoline-8-ol}.

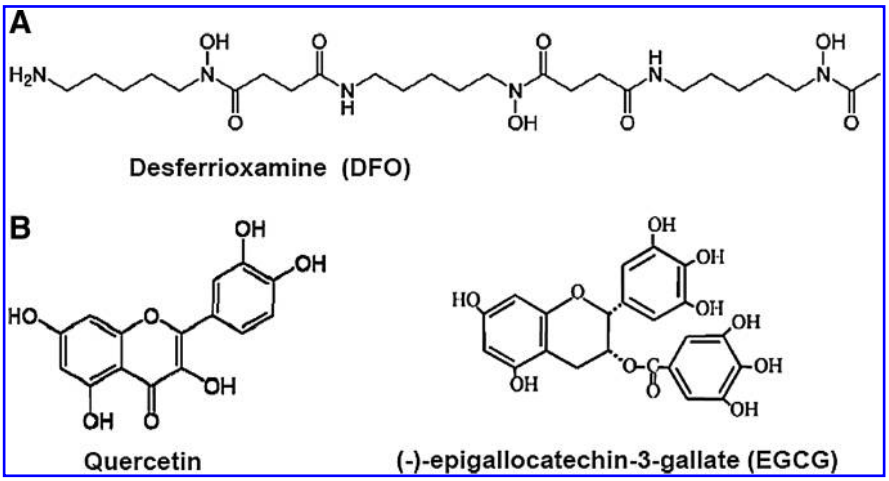

FIG. 6. Chemical structures of natural iron-chelating/radical-scavenger compounds. (**A**) desferrioxamine (DFO). (**B**) Flavonoid-type compounds, quercetin, and the major polyphenolic compound of green tea, (-)-epigallocatechin-3-gallate (EGCG).

Furthermore, in an experimental model of H_2O_2-induced OS/neurotoxicity, the major metabolite of rasagiline, 1-(*R*)-aminoindan, was found to stimulate significantly the activity of the antioxidant enzyme, catalase (16). 1-(*R*)-aminoindan treatment in H_2O_2-damaged neuronal cells was demonstrated to induce mRNA expression levels of various phase II enzymes, which are an important cellular defense against oxidative injury: catalase, which acts to neutralize H_2O_2 toxicity; quinine oxidoreductase 1, a cytosolic protein that reduces and detoxifies quinines, protecting the cells against redox cycling and OS and peroxiredoxin 1, which uses the reactivity of the cysteine residues to reduce H_2O_2 and other peroxides (16). These results suggest that the increase of endogenous antioxidants might be a possible mechanism involved in the neuroprotective effects of 1-(*R*)-aminoindan and thus, may contribute to the beneficial neuroprotective mechanism of action of its parental compound, rasagiline (15).

Chelation therapy was previously introduced as a novel therapy concept and rationale for the development of metal-binding drugs for PD, as detailed in several recent reviews that characterized the therapeutic potential of a range of iron chelators (34, 118, 208, 340). In fact, a number of iron chelators/antioxidants has been shown to possess neuroprotective activity in animal models of PD; for example, the natural prototype iron chelator/radical scavenger, desferrioxamine (DFO) (Fig. 6A) (151), protected against dopaminergic neurodegeneration induced by 6-OHDA (19, 20) iron and MPTP (162, 163). More recently, the antibiotic metal chelator, 5-chloro-7-iodo-8-hydroxyquinoline (clioquinol) was shown to prevent MPTP neurotoxicity in mice (150). However, clioquinol is highly toxic (41), whereas the hydrophilic nature of DFO and its large molecular size may limit it from penetrating the blood–brain barrier (BBB) (252). It was suggested that iron chelators must be carefully controlled to prevent toxicity and side effects (117, 354) and, thus, should be designed to enhance selectively, affinity, stability, renal clearance, and oral activity with maintaining low toxicity (113).

The concept of iron chelators for clinical use in neurologic disorders, which can cross the BBB and possess iron-chelating ability for the removal of excess of iron in the brain, led our group to develop nontoxic, lipophilic, brain-permeable multifunctional drugs for neurodegenerative diseases (357, 358). Based on a multimodal drug-design paradigm, the neuroprotective propargyl moiety of rasagiline (Fig. 5) and selegiline (350) was incorporated into the antioxidant–iron-chelator moiety of VK28 (5-[4-(2-hydroxyethyl) piperazine-1-ylmethyl]-quinoline-8-ol) (Fig. 5) (21, 357, 358). Previously, VK28 was successfully used in the reversal of 6-OHDA–induced detrimental effects in the rat (21) and neural cell models (13).

Novel multifunctional iron-chelating compounds were designed to prevent the ability of iron to induce OS and reactive hydroxyl radical generation through the interaction with H_2O_2, as well as to inhibit the formation of reactive hydroxyl radical from H_2O_2, generated by MAO and to potentiate the pharmacologic action of accumulated DA, formed from L-DOPA. In a series of multifunctional iron chelators, the compound M30 [5-(*N*-methyl-*N*-propargylaminomethyl)-8-hydroxyquinoline] (Fig. 5) was found to be a most-potent iron chelator, displaying highly effective inhibition of MAO-A and MAO-B activities and iron-dependent lipid peroxidation *in vitro* and *in vivo* (88, 89, 357–359). M30 possessed solubility and selective iron-chelation properties (compared with zinc and copper) (358). Neither VK28 nor M30 was cytotoxic, as shown by the genotoxicity assay, performed in three different cell lines, A549, SH-SY5Y, or HepG2; inhibition of cytochrome p450 isozymes and voltage-dependent potassium channel–blocking test (Varinel, Inc. West Chester, PA). Additionally, M30 was found to invoke a wide range of pharmacological activities, including a neurorescue response; a protective potency against OS insults, H_2O_2, and SIN-1 (peroxynitrite generator, 3-morpholino sydnonimine), and a regulatory action on neuronal differentiation and neurite outgrowth in various neuronal cell lines (9–11, 159). In the *in vivo* studies, M30 was shown to prevent the loss of mouse TH-positive neurons, induced by post-intranigral injection of lactacystin (proteasome inhibitor) and significantly to improve behavioral performances and to attenuate inhibition of ubiquitin-proteasome activity, iron increase, and microglial activation in the ipsilateral SN (363). Moreover, we recently demonstrated that M30 prevented MPTP-induced striatal DA depletion (89), as well as restored nigrostiaral DA neurons in the post-MPTP mouse model of PD (90).

Another class of agents that possess free radical scavenging and antioxidant properties, reported to possess neuroprotective effects, includes phenolic and polyphenolic compounds, such as vitamin E and natural catechins (*e.g.*, green tea catechins)

(315, 356). Recently, Perron and co-workers (235, 236) correlated the pK_a and IC_{50} values of phenolic compounds for inhibition of Fe^{2+}/H_2O_2 -induced neurotoxicity, and thus, suggested that the binding of the polyphenols to iron was essential for their antioxidant activity. Polyphenols also can exert prooxidant activities under certain experimental conditions (107, 185). It was suggested that mild prooxidant effects of polyphenols can also be beneficial, because by inducing a mild degree of OS, the level of antioxidant enzyme defenses might be increased, leading to overall cytoprotection (76, 107). A biphasic mode of biologic activity of green tea catechins indicated a concentration-dependent window of pharmacologic action. At high concentrations, green tea catechins exhibited prooxidant and proapoptotic activity, which might be responsible for the anti–cancer-cell death effects, whereas at low doses, they possess neuroprotective effects against a wide spectrum of neurotoxic insults (14, 337). Neuroprotective *in vivo* studies using MPTP have shown that both green tea extract and the major component of green tea, (-)-epigallocatechin-3-gallate (EGCG) (Fig. 6B) exert highly potent activities in preventing mice striatal DA depletion and SN dopaminergic neuronal loss (178). In SH-SY5Y and PC12 cells, EGCG was found to attenuate neuronal cell death induced by 6-OHDA (177, 216).

B. Alzheimer's disease

AD is the most prevalent neurodegenerative disease in the elderly population, and it has been estimated that ~5% of adults older than 65 years are affected by this disease (38). Its predominant clinical manifestation is the progressive memory deterioration and other changes in brain function, including disordered behavior and impairment in language, comprehension, and visual–spatial skills (319). The neuropathology of AD is characterized by several features, including extracellular deposition of amyloid β peptide (Aβ)-containing plaques in the cerebral cortical regions, accompanied by the presence of intracellular neurofibrillary tangles and a progressive loss of basal forebrain cholinergic neurons, leading to reductions in cholinergic markers, such as acetylcholine levels, choline acetyltransferase (ChAT), and muscarinic and nicotinic acetylcholine-receptor binding (277, 281). Additionally, accumulating evidence indicates that many cytotoxic signals in the AD brain can initiate apoptotic processes, including OS, inflammation, and iron accumulation (144, 258, 299). Iron is significantly concentrated around amyloid senile plaques and neurofibrillary tangles (NFTs), leading to alterations in the pattern of the interaction between IRPs and their IREs, and disruption in the sequestration and storage of iron (187, 239). High levels of iron have been reported in the amyloid plaques of the Tg2576 transgenic mouse model for AD, resembling those seen in the brains of AD patients (298). In addition to iron accumulation in senile plaques, it was demonstrated that the amount of iron present in the AD neuropil is twice that found in the neuropil of nondemented brains (239). Further studies suggested that iron accumulation could be an important contributor to the OS damage of AD pathology, and thus, the neurons in AD brains experience a high oxidative load (45, 122, 210, 297). It was found that NFTs and senile plaques contain redox-active transition metals and may exert prooxidant/antioxidant activities, depending on the balance among neuronal antioxidants and reductants (273). Postmortem analyses of AD patients' brains have revealed activation of two enzymatic indicators of cellular OS: heme oxygenase (HO-1) (311) and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (288). Also, HO-1 was greatly enhanced in neurons and astrocytes of the hippocampus and cerebral cortex of AD subjects, co-localizing to senile plaques and NFTs. A previous study reported that ribosomal RNA provides a binding site for redox-active iron and serves as a redox center within the cytoplasm of vulnerable neurons in AD brain, in advance to the appearance of morphologic change indicating neurodegeneration (122).

A number of studies suggested that iron homeostasis is disrupted in AD (58, 59, 187, 296). Thus, abnormal localization of the iron-regulatory protein, IRP2, which was shown in AD, might be linked to impaired iron homeostasis in AD (300). However, this is likely to be a secondary effect through another process, such as increased HO-1 activity in response to cellular OS (276), or a decrease in heme bioavailability resulting from Aβ–binding heme, increasing free-Fe levels (7). In addition, the location of transferrin in senile plaques, instead of its regular location in the cytosol of oligodendrocytes, indicated that transferrin becomes trapped within plaques while transporting iron between cells (59). Ferritin and the mediator of iron uptake by cells, melanotransferrin, are altered and expressed within reactive microglial cells, both in and around the senile plaques (101). Several studies assessing the frequency and association of mutation in the class I–like major histocompatibility complex gene, *HFE* (known to be related to a disorder of excessive iron uptake, hereditary hemochromatosis), with AD, reported on contradictory data [see review (57)].

At the biochemical level, iron was demonstrated to facilitate the aggregation of Aβ and to induce aggregation of the major constituent of NFTs, hyperphosphorylated τ (tau) (39, 46). It was suggested that the toxicity of Aβ is mediated, at least in part, through redox-active iron; neuronal toxicity was significantly attenuated when Aβ was pretreated with the iron chelator DFO, whereas conversely, the toxicity was restored to original levels after incubation of holo-Aβ with excess free iron (264). *In vitro* studies demonstrated that Aβ has high affinity for iron, and the iron-binding sites are located in the hydrophilic N-terminal part of the peptide (8). Another molecular link between iron metabolism and AD pathogenesis was provided by Rogers *et al.* (258, 259), who described the presence of an IRE in the 5′ untranslated region (5′UTR) of the amyloid precursor protein (APP) transcript. Thus, APP 5′UTR is selectively responsive to intracellular iron levels in a pattern that reflects iron-dependent regulation of intracellular APP synthesis. Iron levels were shown to regulate mRNA translation of APP holoprotein in astrocytes (256, 259, 260) and neuroblastoma cells by a pathway similar to iron control in the translation of the ferritin-L and -H mRNAs by IREs in their 5′UTRs (257). Thus, iron chelation has the potential to prevent iron-induced ROS, OS, and Aβ peptide aggregation, and therefore, chelation therapy may be considered a valuable therapeutic strategy for AD (Fig. 7).

1. Therapeutic strategies in AD. Recently, several studies reported a survey of various metal chelators, including iron homeostasis restoring compounds, that are potentially useful for the treatment of AD (34, 118, 208, 340, 353). The iron chelator, DFO, was reported in a single clinical study to slow

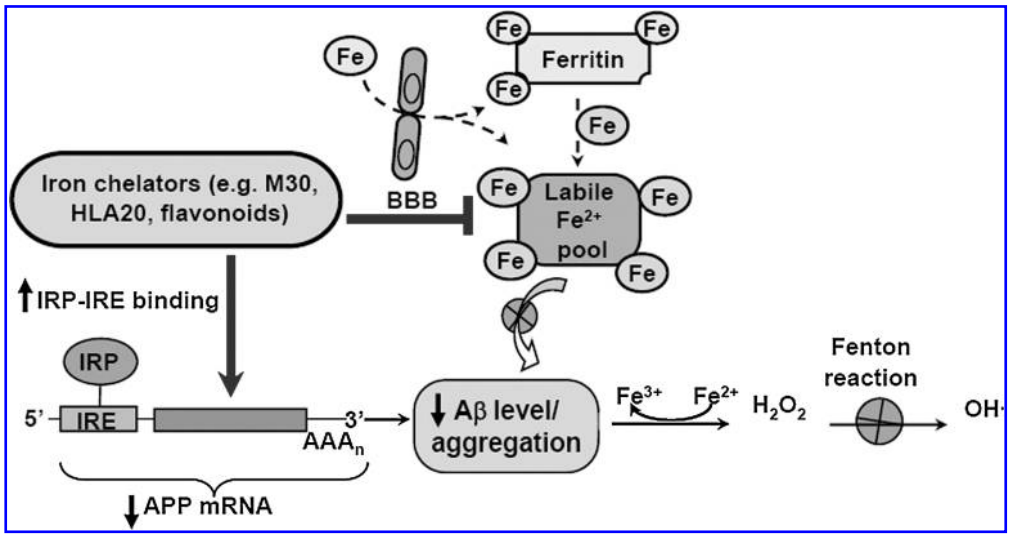

FIG. 7. Proposed schematic model suggesting iron-chelation therapy as a therapeutic strategy for AD. Iron-chelating compounds target the APP 5′UTR, thus possessing the capacity to reduce APP translation and subsequently Aβ-generation levels. A full explanation is discussed in the text.

the progression of AD dementia (61). *In vitro*, DFO was shown to prevent the formation of β-pleated sheets of A$\beta_{1\text{-}42}$ and to dissolve preformed β-pleated sheets of plaque-like amyloid (123). In addition, the metal ligand clioquinol was reported to inhibit Aβ accumulation in an AD transgenic mouse model through its actions as a metal chelator (52). A second-generation, 8-hydroxy quinoline derivative of clioquinol (PBT2) also was found to restore cognition in Alzheimer transgenic mice, in association with decreased interstitial Aβ (1). The mechanism of action of clioquinol was suggested to be through metal sequestration, resulting in Aβ dissolution. However, clioquinol could also act by modulation of cellular biometal metabolism and APP expression/processing (316). Alternatively, it was proposed that clioquinol induced neuroprotection by enhancing intracellular Cu^{2+} and Zn^{2+} uptake, thereby acting as an ionophore that favors the clearance of Cu/Zn from the amyloid plaques and synaptic space (339). Clinical studies demonstrated that both clioquinol and PBT2 can be safely used in AD patients and can attenuate some of the AD-associated cognitive deficits (40, 53, 164, 255, 353). However, long-term treatment with clioquinol was reported to be limited by an adverse side effect (41).

The identification of an IRE in the 5′UTR of the APP transcript led to a novel therapeutic approach aimed at reducing amyloidosis by FDA preapproved drugs targeted to the IRE in the APP mRNA 5′UTR (211, 233). For example, DFO, tetrathiomolybdate (Cu^{2+} chelator) and dimercaptopropanol (Pb^{2+} and Hg^{2+} chelator) were found to suppress APP holoprotein expression and to reduce Aβ peptide secretion (211, 233). In addition, the bifunctional molecule XH-1, which contains both amyloid-binding and metal-chelating moieties, was shown to reduce APP expression in SH-SY5Y cells and to attenuate cerebral Aβ in presenilin (PS1)/APP transgenic mice model of AD (69). Additional drug classes also were reported to suppress the APP 5′UTR and to limit APP expression, including, antibiotics, selective serotonin reuptake inhibitors (SSRIs), and other selective receptor antagonists and agonists (233). Similarly, the novel iron chelator, VK28, and the multifunctional iron chelating drug M30 (358) were found to suppress translation of a luciferase reporter mRNA through the APP 5′UTR sequence (9). This effect may account, at least in part, for the observed downregulation of membrane-associated holo-APP levels in the mouse hippocampus and *in vitro* in SH-SY5Y neuroblastoma cells, presumably by chelating intracellular iron pools (9, 11). Furthermore, M30 markedly reduced the levels of the amyloidogenic Aβ in the medium of CHO cells, stably transfected with the APP "Swedish" mutation (CHO/ΔNL) (9, 11) and protected primary cultured neurons against Aβ toxicity (10).

Furthermore, an increase in MAO-B activity within reactive microglia in AD brain tissues is considered to contribute to high levels of H_2O_2 formation, as a by-product of amine turnover (272). The multimodal drug, ladostigil (TV3326) [(*N*-propargyl-(3R) aminoindan-5yl)-ethyl methyl carbamate], which combines in a single molecule the neuroprotective, antioxidative, and selective MAO-B inhibitory activities of rasagiline with the cholinesterase (ChE) inhibitory activity of the anti-AD drug, rivastigmine (338), was recently demonstrated to regulate the APP-processing pathway (346). Ladostigil dose-dependently increased cell viability, associated with an increase in catalase activity and a decrease of intracellular ROS production in SH-SY5Y cells exposed to H_2O_2 (335). These findings indicate that ladostigil possesses protective effects against OS-induced neuronal apoptosis, which might be beneficial for treatment of OS in aging and AD.

Naturally occurring polyphenols (*e.g.*, EGCG and curcumin) might be used as another novel and promising therapeutic approach for treating AD. Both compounds possess antioxidant/prooxidant and metal-chelating (iron and copper) activities (14, 102, 158) and have been demonstrated to exert neuroprotective activity against a variety of neurotoxic insults, as well as to regulate APP processing and Aβ burden in cell culture and *in vivo* (176). EGCG treatment led to a reduction of both the mature and full-length cellular holo-APP levels (without altering APP mRNA) in SH-SY5Y cells and of Aβ levels in CHO/ΔNL, transfected with human

APP695 containing the "Swedish" mutation (APPsw,Lsy670/Asn, Met671/Leu) (250). The reduction in APP protein after EGCG treatment was blocked by exogenous iron, thus providing further support to the implication of metal chelation (250). Additionally, prolonged administration of EGCG to mice induced a reduction in holo-APP levels in the hippocampus (11). In AD "Swedish" mutant APP-overexpressing mice (APPsw Tg), EGCG significantly reduced cerebral Aβ levels and amyloid plaques and promoted the generation of the soluble, nonamyloidogenic form of APP (sAPPα) through activation of α-secretase cleavage (248). In accordance, metallopeptidase 10 (ADAM10) activation was found to be required for EGCG-induced α-secretase cleavage of APP (220). Recent studies further demonstrated that EGCG reduced Aβ-mediated cognitive impairment and modulated Tau pathology in APPsw Tg mice (247).

C. Amyotrophic lateral sclerosis

ALS, commonly referred to as Lou Gehrig disease, is a relentlessly progressive, neurologic disorder with an estimated prevalence of four to six cases per 100,000 (63). The onset of ALS is most common in midlife (usually between ages 45 and 60), with a typical disease course of 1 to 5 years. The majority of ALS cases (90%) are of unknown etiology and are classified as sporadic. The remaining 10% of cases are familial, and six disease-causative genes from 11 loci have been identified by linkage analysis and positional cloning (322). Both sporadic and familial forms of ALS are clinically and pathologically similar, suggesting a possible common pathogenesis and final pathway of neurodegeneration. The molecular pathogenesis of ALS is poorly understood, contributing to the lack of effective system-based therapies to treat this disease. Investigations have implied that ALS is a multifactorial and multisystemic disease that arises through a combination of several mechanisms act by concurring damage inside motor neurons and their neighboring nonmotor cells, including protein misfolding and aggregation; genetic factors; OS damage and mitochondrial dysfunction; defective axonal transport; excitotoxicity, and neuroinflammation (60, 321).

An early indication for a role of iron in the pathogenesis of ALS was provided by the elevated iron levels in the CNS of both sporadic and familial forms (127, 147, 219). In addition, the expression of ferritin was induced at the last stages of the disease in the SOD1-G93A transgenic mouse (which develops symptoms and pathology similar to those of ALS patients), indicating high iron concentrations (60). In line with this, it was reported that transferrin is localized in Bunina bodies of spinal cord neurons from ALS patients (68, 207), suggesting the involvement of transferrin in the formation of these inclusions. Interestingly, in transgenic mice expressing the wild-type SOD1 or SOD1-active mutant enzyme, G93A-SOD1, the expression of TfR and IRP1, a positive transcriptional regulator of TfR, were positively modulated in response to increased SOD1 mutation (68). Jeong and collaborators (139) recently described dysregulation of iron-homeostasis mechanism in the CNS in the G37R-SOD1 transgenic mice model of ALS, suggesting that iron-chelator therapy might be useful for the treatment of ALS.

A defect in the *HFE* gene, which was previously associated with the iron-overload diseases, hemochromatosis and AD, is currently associated with ALS (308). The protein normally made by the *HFE* gene is thought to limit the uptake of iron by cells, to protect against OS, and possibly to dampen inflammatory reactions. An increased incidence of the HFE mutation was reported in ALS patients (328). The presence of this mutation was shown to disrupt expression of tubulin and actin at the protein levels, potentially consistent with the disruption of axonal transport seen in ALS and also associated with a decrease in SOD1 expression (328).

1. Chelation therapeutic strategy in ALS. Treatment of ALS has been fuelled in part by frustration over the shortcomings of the symptomatic drugs available, because these are incapable of slowing the progression of the disease and neuronal degeneration. Regrettably, the single drug approved for use in ALS, riluzole, a membrane-stabilizing drug, only slightly prolongs survival (204). Currently, >150 different potential therapeutic agents or strategies have been tested in transgenic ALS mice, according to published trials (320). This list involves 108 pharmacotherapies, 14 gene or antisense therapies, nine cell transplantations, three immunizations, and seven dietary or lifestyle regimens. The pharmacotherapy spectrum encompasses antioxidants, antiexcitotoxins, antiaggregation compounds, antiapoptotics, antiinflammatories, and neurotrophic agents. Unfortunately, therapeutic modifiers of murine ALS have failed to translate in patients successfully, probably because most of these trials tested single agents that affect only one mechanism or because of delivery limitations. Given the multiplicity of pathologic mechanisms implicated in ALS, new ALS therapies may consider a simultaneous manipulation of multiple targets.

Combination treatments or polypharmacy targeting different disease mechanisms have consistently shown superior efficacy in transgenic ALS mice (64, 157, 326). Based on this reasoning, our group recently demonstrated that the multifunctional iron-chelating drugs, M30 and HLA20, possess neuroprotective pharmacological activities in NSC-34 cells, a widely used mouse motor neuron hybrid cell line, including improvement of neuronal survival, promotion of neuronal differentiation, and induction of the PKC and MAPK signaling pathways (159). In addition, this study showed that treatment with M30 provided clear benefits in G93A-SOD1- ALS mice, significantly increasing their survival and delaying the onset of neurologic dysfunction, even when the treatment was initiated at a relatively advanced stage of the disease (159). These results may be of significant relevance for further development of M30 for the treatment of ALS. Considering the neuritogenic effect of M30, demonstrated in motoneuron NSC-34 cells (159), it is possible that the *in vivo* action of M30 is mediated through the regeneration process of motor nerves, inducing neurodifferentiation and sprouting of axons, leading to reinnervations of muscle fibers.

III. Iron, HIF-1 System, and Neuroprotection

Neuroprotection by iron-chelating agents has been widely attributed to their ability to prevent iron involvement in the redox cycling and thereby to inhibit hydroxyl radical formation by the Fenton or Haber-Weiss reaction. The next section addresses an additional level of neuroprotection by iron

chelators, which involves inhibition of iron-dependent HIF-PHD enzymes, resulting in the induction of HIF-1 expression and transcription of HIF-1–related downstream genes that may contribute to survival in the CNS. Previous reports described the diversity of cellular responses to iron chelators, suggesting that these pluripotent neuroprotective compounds may enhance neuronal survival by a mechanism other than simply diminishing hydroxyl radical formation and OS by sequestering redox active iron or inhibiting iron-dependent lipid peroxidation [see reviews (112, 291)].

A. HIF/PHD pathway and HIF-target genes

HIF-1 is a central transcriptional regulator of hypoxia-inducible genes and a member of the basic helix-loop-helix Per/Aryl hydrocarbon-receptor translocator (ARNT)/Sim (PAS) family, consisting of one of the three alternative oxygen-regulated α-chains (HIF-1α, -2α, and -3α) and of the three stable β-chain family members (HIF-1β/ARNT1, HIF-2β/ARNT2, and HIF-3β/ARNT3a) (145). HIF-α members exclusively dimerize with -β family members, whereas the β subunits can dimerize with non-HIF transcription factors, each encoded by different genes (29, 240). HIF-1α was first identified *in vitro*, through its property of DNA-binding activity of the 3′ EPO gene enhancer under hypoxic conditions, in which its concentration and activity increased exponentially when oxygen tensions were decreased over physiologically relevant ranges (285, 327). Both HIF-1α and HIF-2α both contain two constitutive transactivation domains located in their C-terminal halves, called the N-terminal transactivation (N-TAD) and C-terminal transactivation (C-TAD), and can activate transcription when bound to DNA in complex with a β subunit (145) (Fig. 8). HIF-α subunits are highly unstable and were initially identified to be degraded in the presence of oxygen through asparaginyl and prolyl hydroxylation (attachment of an OH groups) by one of the three conserved members of the egg-laying–defective nine (EGLN) family, called HIF-α-PHDs (EGLN1/PHD2; EGLN2/PHD1; EGLN3/PHD3) (29). The PHDs belong to a superfamily of iron-dependent dioxygenase, whose activity requires molecular oxygen, as a substrate, and 2-oxoglutarate, as a cosubstrate to hydroxylation, thereby producing carbon dioxide and succinate (29).

The three isozymes of the mammalian PHDs, PHD1, 2, and 3, present in the brain (75), have been identified and reported to share homology in the C-terminal catalytic domain, but have significant differences in their N-terminal sequence (290, 291). These PHDs also differ in regulatory expression, tissue distribution, cellular localization, and their effect on HIF-1α hydroxylation (290, 291). Although the HIFs are the most commonly studied substrates for PHDs, other known substrates exist, including CREB, IRP2, the NF-κB partner IKKβ, and the Rbp1 subunit of RNA polymerase II complex (47, 65, 292). It was suggested that each of the PHD isoforms regulates distinct specific downstream targets/transcription factors that contribute to the overall neuroprotection. Consequently, PHDs might mediate neuroprotection in an HIF-independent fashion (292). For example; PHD3 was demonstrated to be specifically involved in NGF-withdrawal–induced neuronal apoptosis (172). A recent report suggests that Rpb1 is a possible HIF-independent target for PHD1 and functions as a crucial modulator of gene expression associated with increased resistance to OS in cell culture (203).

PHD2 was shown to be the enzyme with the strongest and most direct impact inhibition on HIF-1α stability and HIF-1–target genes under normoxic conditions, when sufficient oxygen is present (30). The catalytic site of these enzymes contains conserved 2-histidine-1-carboxylated coordination motif for the ligand Fe^{2+}, which in turn binds the oxygen and 2-oxoglutarate (195, 200). A recent study demonstrated a high stringency for the iron-binding residues in the PHD active site (97). In addition, a cofactor ascorbate is required after repeated hydroxylation reaction cycles to prevent the autooxidation of the enzymes (29). HIF-1α is enzymatically hydroxylated on one of two potential prolyl residues (Pro402 and Pro564) within HIF-TAD, the oxygen-dependent degradation domain (ODDD), and thus, generates a pVHL-binding site (145). In addition to prolyl-hydroxylation, lysyl-acetylation was found to be another mechanism underlying HIF-1β stability. The acetylation of a lysine residue (K532) within the ODDD also enhances the interaction of pVHL and HIF-1β (138). Because pVHL acts as part of an E3-ubiquitin ligase complex, HIF-1β is ubiquitinylated and marked for proteasomal degradation.

Under normoxic conditions, the HIF-1α protein subunits are constitutively expressed, but the ODDD is recognized by the product of the pVHL tumor-suppressor gene that tags the subunit with polyubiquitin to promote HIF-1α degradation by the proteasome (36, 199) (Fig. 8). It was suggested that either the N-terminal site or the C-terminal within ODDD participates in the iron-dependent regulation of HIF-1α, but the C-terminal site alone participates in the oxygen-dependent regulation, which requires both the lysyl-acetylation and prolyl-hydroxylation (170). In addition, during normoxia, oxygen plus 2-oxoglutarate activates an asparaginyl hydroxylase, which is also called factor-inhibiting HIF (FIH) (245). Like prolyl hydroxylation, HIF asparaginyl hydroxylation also is catalyzed by a member of the 2-oxoglutarate–dependent oxygenase superfamily, and its catalytic domain harbors Fe^{2+} ions, but differs from the PHDs sequence (279). This enzyme hydroxylates an asparagine residue (Asn803) in HIF-1α protein and subsequently changes the conformation of the protein so that it cannot bind p300 coactivator, which in turn prevents HIF-1α binding to hypoxia-response elements in the promoters of HIF-1–target genes. Therefore, asparaginyl hydroxylation provides a second oxygen-regulated mechanism by which HIF-1α molecules that escape the prolyl-hydroxylation/degradation pathway are prevented from activating transcription through the p300 coactivator (Fig. 8). Thus, normoxia causes HIF-1α degradation and blocks HIF-1 transcriptional activation (245).

Under hypoxia-mimetic conditions, or iron-depletion environments, pVHL fails to recognize the HIF-1α subunit and, thus, the α-subunit is stabilized and subsequently translocated into the nucleus, allowing HIF-1α to accumulate (36, 129). Once in the nucleus, HIF-1α dimerizes with the β-subunit and interacts with p300 coactivator; this complex binds to the pentanucleotide HREs, RCGTG within the promoters of HIF-1–target genes, and initiates the induction of their expression levels (36, 290) (Fig. 9).

Additionally, a variety of HIF-1 stimuli, such as cytokines, growth factors, nitric oxide, iron chelators, and divalent cations, function independent of oxygen concentration by inhibition of PHDs and HIF-1α degradation (12, 29, 290). For example, nickel and $CoCl_2$ can potentially replace iron at the catalytic site of PHD enzymes and thus inhibit these enzymes,

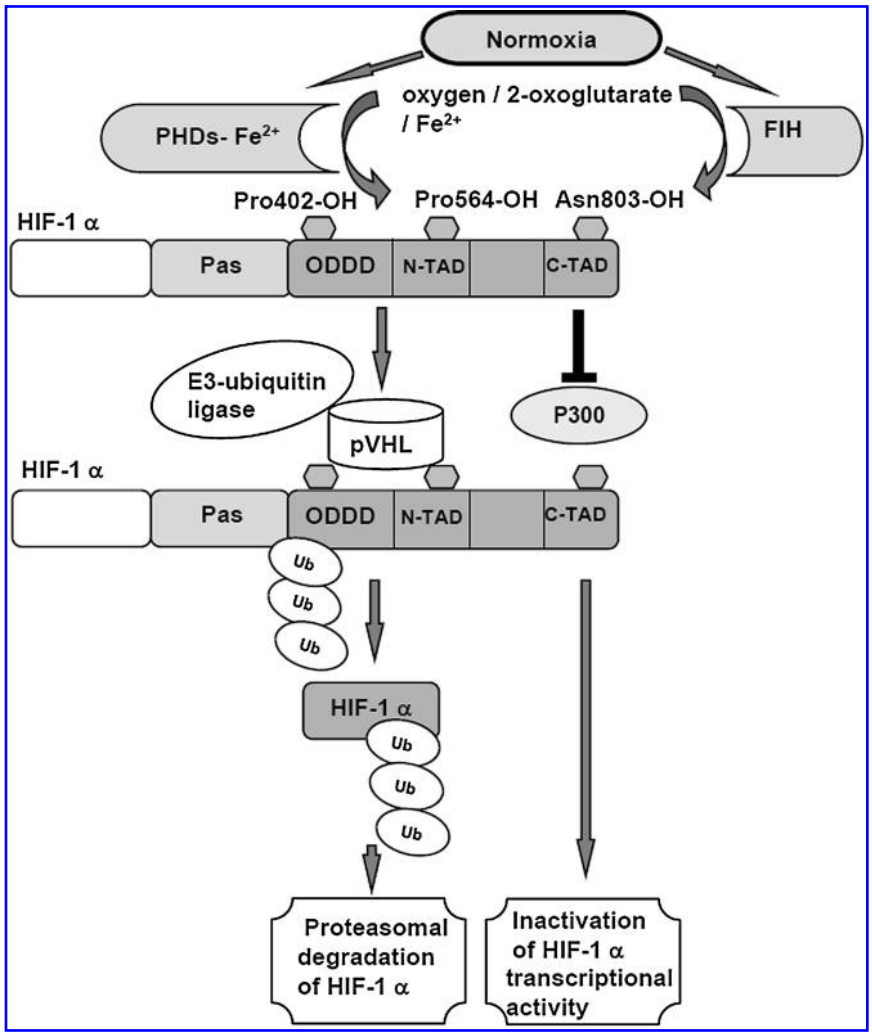

FIG. 8. Schematic diagram illustrating the regulation of HIF-1α under normoxia conditions. HIF-1 is a heterodimeric α,β-transcriptional complex that mediates the cellular response to oxygen availability in multicellular organisms. The HIF-1α subunit is rapidly degraded by the proteasome in the presence of sufficient dioxygen (anoxia) in a process involving the posttranslational hydroxylation of two conserved prolyl residues in HIF-1α. The von Hippel–Lindau tumor-suppressor protein (pVHL) enables binding of the prolyl-hydroxylated HIF-α to a ubiquitin E3 ligase complex that catalyzes ubiquitinylation and degradation of HIF-1α by the ubiquitin–proteasome pathway. Conversely, factor-inhibiting HIF (FIH) hydroxylates an asparagine residue in the carboxy-terminal activation domain, which blocks p300 coactivator recruitment and results in the inactivation of HIF-1α transcriptional activity.

produce hypoxia-like response, and enhance the expression of HIF-1–dependent genes (197, 269). The molecular mechanisms through which these PHDs inhibitors may promote tolerance to ischemic insults have been reviewed by Harten and co-workers (112). Moreover, chemically mimetic stimuli, which are distinct from the hypoxic stimulus, can regulate HIF-1 translation on the already expressed α-subunit (*e.g.*, cytokines and IGFs were shown to induce activation of HIF-1α through MAPK regulation) (114, 283), which participates in phosphorylation of PI3K/Akt signaling pathways (124, 360) (Fig. 10). PKC was demonstrated to increase the rate of HIF-1α transcription and functions in conjunction with the PI3K/Akt signaling pathways by phosphorylating the S6 ribosomal protein, which specifically recognizes and enhances the mRNA transcripts of HIF-1α (364) (Fig. 10). Additionally, the bacterial lipopolysaccharide was shown to induce HIF-1α mRNA expression through activation of NF-κB site in the promoter of the HIF-1α gene in monocytes (84). In general, HIF-1α protein expression can be regulated at both transcriptional and posttranscriptional levels that determine the rate of HIF-1α mRNA synthesis, stability, degradation, and binding ability (266).

The overall beneficial molecular and biochemical stimulation can mediate upregulation of HIF-1α and then activate transcription of a large number of target genes. These HIF-1–target genes would affect, for example, glucose and lactate delivery; increase blood flow, promote glycolysis, as well as many other neuroprotective processes (79, 148). More than 100 genes are known to have hypoxia-response elements in their promoters and to be activated by hypoxia and HIF-1 (77). These include genes that are involved in hematopoiesis (*e.g.*, EPO), iron metabolism (*e.g.*, transferrin, TfR), angiogenesis and vascularization [*e.g.*, VEGF, Flt-1 (VEGF receptor 1), endoglin, plasminogen activator inhibitor-1, ADM, endothelin-1, HO-1, NOS-2], energy metabolism (*e.g.*, GLUT-1, enolase 1, ALDA, hexokinase 1, 2, and 3), antioxidant enzymes (*e.g.*, Mn-SOD), cell proliferation and differentiation [*e.g.*, IGF binding proteins 1 and 3, transforming growth factor (TGF)-β, cyclin G2, opioid growth factor (OGF)-2, caspase 9], pH regulation (*e.g.*, carbonic anhydrase), neurotransmitter synthesis (e.g., TH), and matrix metabolism (*e.g.*, collagen prolyl-4-hydroxylase-α1) (26, 167, 198, 282, 293, 327, 352). A major support for this target-gene regulation was the finding that cells lacking pVHL produced high levels of HIF-1–responsive mRNAs, such as the VEGF and GLUT-1, under both normoxic and hypoxic conditions (126). Recently, it was suggested that low-molecular-weight inhibitors of HIF-PHDs, which activate HIF-1α, and subsequently HIF-1–target genes could mimic, in part, the protective effects of exposure to hypoxia (6, 112, 246, 293, 352). Specifically, Siddiq *et al.*,

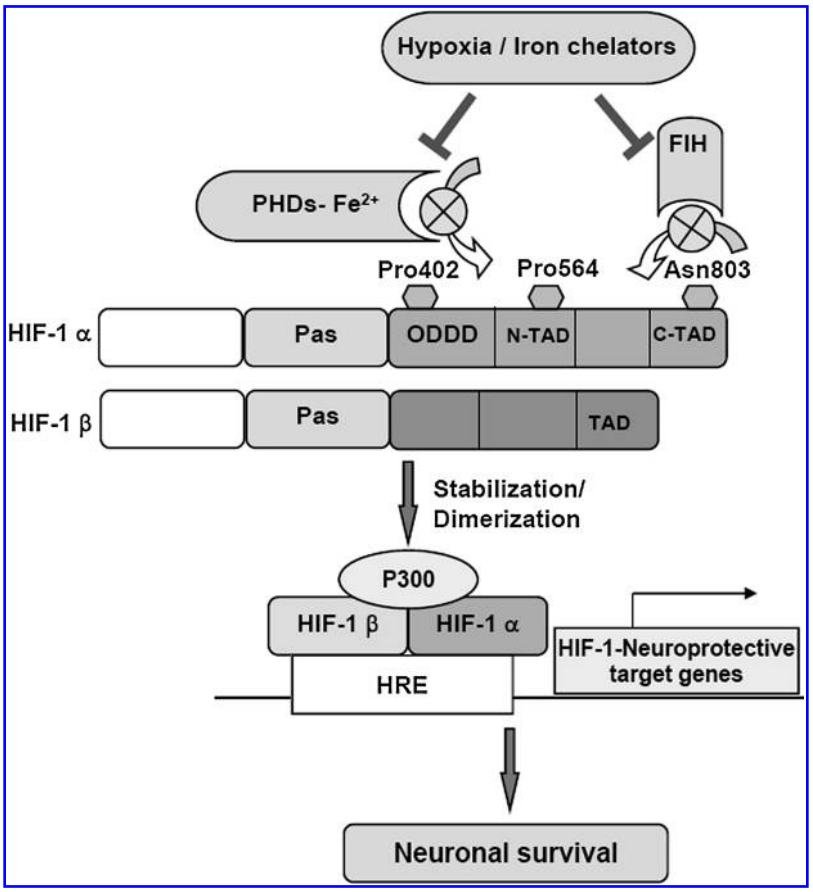

FIG. 9. Schematic diagram illustrating the regulation of HIF-1α under hypoxia and iron-chelating conditions. During hypoxia or when PHDs are inhibited pharmacologically (*e.g.*, iron chelators), HIF-1α is not degraded and translocates to the nucleus, where it binds to the HIF-1β subunit. Interaction of HIF-1α and HIF-1β heterodimers with the p300 coactivator initiates binding to HIF-DNA consensus-binding sites, resulting in increased transcription of HIF-1–target genes involved in the adaptation and protection of neuronal cells.

(292) suggested that the HIF-PHD inhibitors, dimethyl-oxalylglycine (DMOG) and 3,4-ethyl 3,4-dimethylbenzoate (DHB), can stabilize three transcriptional activators (HIF-1, 2, and CREB), that can bind to cognate hypoxia-response sequences to transactivate adaptive genes. Yet, it was demonstrated that global HIF-PHD inhibitors could protect neurons even when HIF-1α and CREB were suppressed (292).

It should be noted that, depending on the specific context, HIF induction may also have detrimental effects. Thus, it was found that the stable expression of HIF-1α potentiated cell death induced by glutamate toxicity, but protected cells from endoplasmic reticulum stress-induced and DNA damage–induced death (5).

B. Hypoxic preconditioning

Reduced oxygen supply (hypoxia) or reduced blood flow (ischemia) to the brain is a major cause of morbidity and mortality in both perinatal and adult periods, often resulting in cognitive impairment, seizures, and other neurologic disabilities (313). Perinatal hypoxia–ischemia (HI) from a variety of causes is responsible for significant morbidity and mortality in term infants. The immature brain is more susceptible to HI, secondary to immaturity of vascular regulation and maturational differences in both metabolic function and free radical generation and scavenging in selectively vulnerable areas that depend on age at insult (108, 325). The neonatal brain is particularly vulnerable to OS because of its high concentration of lipids, high rate of oxygen consumption, altered balance of antioxidants, and increased availability of non–protein-bound free iron (227, 263). Indeed, it was shown that iron content of murine hippocampus is fourfold higher than that in the cortex, potentially explaining the selective vulnerability of this region in neonatal mice (271).

The neuroprotective concept of preconditioning is based on the observation that a brief noninjurious episode of ischemia is able to protect the brain from a subsequent longer ischemic insult (72). Several distinct preconditioning stimuli can induce tolerance to ischemic brain injury; among them are noninjurious ischemia, cortical spreading depression, brief episode of seizure, exposure to anesthetic inhalants and a low dose of endotoxin, hyperthermia, or heat shock (154). *In vitro,* ischemic tolerance also was described in neuronal cultures by mimicking the ischemic insult with sublethal oxygen and glucose deprivation (183).

As hypoxic preconditioning is noninvasive and reproducible, it has been widely used to study the mechanisms protecting the brain against global HI, particularly in newborn rats and against focal transient and permanent cerebral ischemia in adult mice (25). However, although HI animal models have increased our understanding of the processes leading to cell death; there are still no pharmacological treatments available to reduce cell death in the ischemic brain (28). Previous studies suggested that hypoxic stress, elicited by ischemia, could constitute one of the initial stimuli inducing adaptive mechanisms that promote endogenous recovery after stroke (287). Thus, it was demonstrated that hypoxia preconditioning–induced tolerance to cerebral ischemia is in association with an increased expression of HIF-1α– and HIF-1–target genes, such as EPO and VEGF (27, 96).

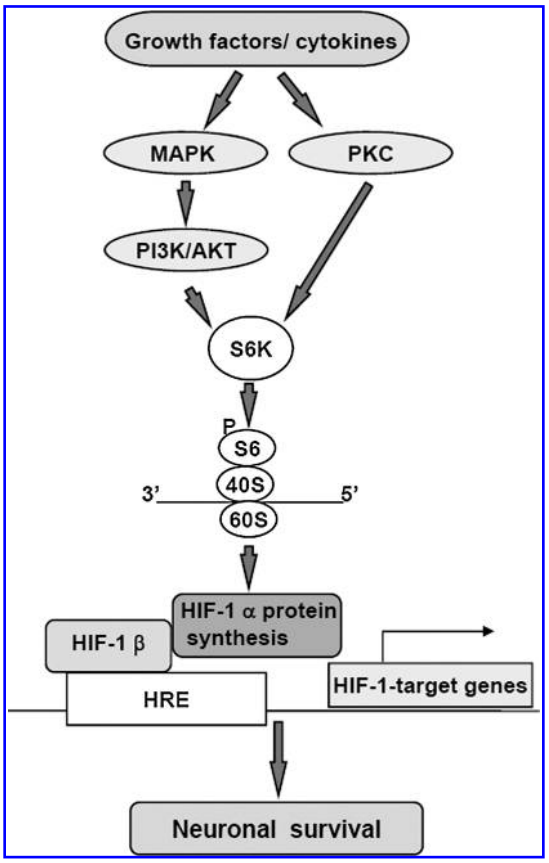

FIG. 10. Schematic diagram illustrating the regulation of HIF-1α by growth factors and cytokines. Alternative activation of HIF-1α is through the phosphatidylinositol-3-kinase (PI3K) and the mitogen-activated protein kinase (MAPK) signaling pathway by mimetic stimuli, such as insulin-like growth factors (IGFs) and cytokines. The protein kinase C (PKC) increases the rate of HIF-1α transcription and functions in conjunction with these signaling pathways.

The adaptation to hypoxia is suggested to be mediated by a cellular system associated with HIF-1, which is a master regulator of oxygen homeostasis (83). The cerebral cortex and hippocampus in newborn rats were demonstrated to be a major site of HIF-1α mRNA and protein expression together with its target genes [*e.g.*, EPO, VEGF, and inducible NOS (iNOS) (26, 295)]. Additionally, several studies demonstrated that HIF-1α– and HIF-1–target genes were induced in the brain after focal ischemia (26), and thus, it was suggested that HIF-1 activation might contribute to the neuroprotective brain preconditioning, which could be used in high-risk deliveries and other clinical situations (25, 27, 234).

In vivo studies with both neonatal and adult rats have shown that preconditioning with the iron chelator, DFO, or the competitive inhibitor of iron, $CoCl_2$, is protective in the preclinical model of ischemia (142, 180, 241). The development of novel small-molecule inhibitors of PHDs, which can induce HIF-1α, has been proposed as a potentially therapeutic strategy for the treatment of ischemic diseases, including stroke. For example, it was demonstrated that the PHD inhibitor compound A (Fibrogen), which prevents iron from acting as a cofactor, induced HIF-1 in a rat model of ischemic stroke (293). In addition, the 2-oxoglutarate analogue, DHB (192), has been shown to induce HIF-1α and to reduce infarct volume in murine brain in a model of focal ischemia (112). These studies and others suggested that PHD inhibition and HIF stabilization may reduce glucose oxidation, thereby reducing OS and hence promoting survival in brain preconditioning (83, 152).

C. HIF-1 pathway and neurodegenerative diseases

In addition to its essential role during embryogenesis, HIF-1–mediated signaling pathways have been suggested to be involved in several brain pathologies (287), although many more studies are still needed to fully clarify the impact of HIF pathway on neurodegenerative pathogenesis. Indeed, the complexity of the contribution of HIF signaling cascade to the progression of neurodegenerative disorders was recently discussed (221), suggesting that it may greatly depend on whether HIF is the cause or the consequence of the disease progression.

During aging, HIF-1α accumulation and signaling-pathway activation in the brain, in response to hypoxia and ischemia, are attenuated (48). Thus, this impairment of HIF-1 signaling in the aged brain may render this brain more susceptible to neurodegenerative processes. In addition, in the AD brain, it was demonstrated that HIF-1 levels were further reduced, compared with age-matched controls (184), and the glucose transporters, GLUT-1 and GLUT-3 were decreased, most probably as the result of reduced HIF-1 levels (184). In the pathogenesis of PD, PHD has been identified as one of the genes that are regulated in the brains of sporadic PD patients (100). Recently, it was demonstrated that PHD inhibition enhanced DA release in the rat brain *in vivo*, suggesting HIF-1α induction (343). Additionally, HIF-1α activation was implicated as a general neuroprotective mechanism against OS and Aβ peptide neurotoxicity (303). It was shown that neuronal cell lines and primary cortical neurons, which were resistant to Aβ-toxicity, revealed an enhanced flux of glucose through the glycolytic and pentose-phosphate pathway, due to induction of HIF-1α protein levels and activity (303). Moreover, deletion of the HRE in the promoter region of the HIF-1–target gene, VEGF, can cause motor degeneration in mice (161). However, VEGF levels were reported to be normal in the ALS spinal cord, but were shown to be low in the blood of ALS patients (218). In AD brain, VEGF expression has been observed in reactive astrocytes and perivascular deposits, implicating the presence of regulatory mechanisms compensating for insufficient vascularity and reduced cerebral perfusion (70). Additionally, the accumulation of VEGF within amyloid plaques in the brains of AD patients has been suggested to result in sequestration and local deficiency of available VEGF (70). Altogether, the regulation of the redox state, as well as the levels of proteins, enzymes, and target genes of the HIF-1 system, has been suggested as a novel subdiscipline in molecular pharmacology, which has promising therapeutic implications in neurodegenerative diseases.

IV. Iron-Chelating Compounds; Neuroprotection Through HIF-1 Activation

Iron plays an essential role in the brain, where it is required as a cofactor for various enzymatic processes, but its excess is a key component in the control of damaging effects,

including hypoxia and oxidative metabolism. Given the links between oxygen transport and iron metabolism, associations between the physiology of hypoxic response and the control of iron availability are important. Several HIF-1–target genes (*e.g.*, transferrin, TfR, and HO-1) are involved in iron homeostasis, reflecting the molecular links between oxygen homeostasis and iron metabolism. In support, a previous report established an association between oxygen and iron regulation by showing that hypoxia results in higher iron absorption (202).

In addition, the PHD and FIH enzymes, which contain a non–heme-bound Fe^{2+} in their catalytic center (29), represent important enzyme families that require iron as an essential cofactor. Intracellular chelatable iron, which belongs to the labile iron pool, can act as a cofactor for their enzyme activity. Thus, it can be predicted that shortage of intracellular iron will result in low PHD activity and upregulation of HIF-1 activity. Iron chelators can inhibit PHD and FIH by preventing the oxidation of Fe^{2+} to Fe^{3+} (Fenton reaction) in their catalytic center, and thus, build up HIF-1α. The activation of HIF-1–related genes is a supplementary important physiologic neuroprotective mechanism, which includes the induction of angiogenesis and activation of the glycolytic metabolism, results in increased delivery of oxygen and nutrition that are crucial for neuronal survival (152). Previous findings provided strong evidence confirming the connection of HIF-1α and iron, demonstrating that pVHL-mediated regulation of HIF-1α stability is iron dependent (199). In addition, it was suggested that a pathway for iron-mediated degradation of IRP2 is required for the activity of PHDs (111). The PHD inhibitor, DMOG, which upregulates HIF-1, was shown to inhibit iron-dependent degradation of IRP2 efficiently only in cells pretreated with DFO (111). Iron chelators were shown to exert their neuroprotective effects, in part, by activating a signal-transduction pathway leading to increased expression of genes known to compensate for hypoxia or OS (293, 352). These findings indicate that iron is a necessary cofactor in the posttranslational modification of HIF.

More recently, iron starvation of C57BL mice resulted in a strong decrease in mRNA of a key regulator of iron absorption and homeostasis, hepcidin, accompanied by a clear increase in HIF-1α levels (238). This study, demonstrating the influence of dietary iron depletion on HIF-1α expression and the control of iron metabolism and homeostasis regulators by the pVHL/HIF pathway, provided essential molecular evidence for the coordination between the process of iron uptake and hypoxic response (238).

Table 1 summarizes the regulation of the HIF-1 system by neuroprotective compounds possessing iron-regulating properties in various neuronal cells and *in vivo* in animal brains; DFO was demonstrated to confer neuroprotection in association with HIF-1α induction in various models of hypoxic–ischemic brain injury in rats and mice (25, 212, 228, 271) and cultured neurons exposed to various insults, such as oxygen and glucose deprivation and H_2O_2 (Table 1) (3, 108). Blocking of HIF-1α by transfection of anti-HIF-1α, reduced DFO neuroprotective effects in hippocampal neurons from E16 CD1 mice, suggesting that the mechanism of action of DFO may involve iron chelation–related HIF-1α protein induction (108). Previous studies demonstrated that DFO treatment led to deficiency in iron-reducing capacity, resulting in inhibition of PHD activity, induction of HIF-1α stabilization and downstream HIF-1–target genes associated with iron homeostasis and metabolism, such as HO-1, TfR, and transferrin (171, 262), and accompanied by a decrease in mitochondrial ROS production in primary neurons (Table 1) (32, 121, 293, 352). Further support for this interesting therapeutic approach came from a study demonstrating that prolonged DFO treatment significantly decreased the size of rat-brain damage after ischemia and improved behavioral recovery (85). In neuronal stem cells, DFO increased HIF-1α protein expression (~100-fold) and subsequently the HIF-1–target gene, VEGF, as well as the brain-derived neurotrophic factor (BDNF) and stromal-derived factor (CXCR) (55). In addition, the involvement of DFO in activating downstream HIF-1–target genes was correlated with its ability to enhance DNA binding of HIF-1α and activating transcription factor 1 (ATF-1)/cAMP response element in cortical cultures and in the H19-7 hippocampal neuronal cell line (293, 352). It was demonstrated that mRNA, protein, and/or activity of genes, whose expression is known to be regulated by HIF-1 (*e.g.*, glycolytic enzymes, $p21^{waf\ 1/cip\ 1}$, TH, and EPO) were increased in cultured cortical neurons in culture in response to DFO (293). Regarding the effect of DFO on the rate-limiting enzyme in catecholamine biosynthesis, TH (Fig. 4), which is also an HIF-1–target gene (217, 278), it was recently reported that DFO produced a delayed increase in the hypoxic ventilatory response, suggesting a gene effect of this iron chelator (215). Moreover, previous studies showed the neuroprotective effect of DFO in animal and cell-culture models of cerebral ischemic stress, associated with EPO upregulation through stabilization of HIF-1α (108, 180, 241, 246).

The liposoluble iron chelator 2,2′-dipyridyl has been shown to reduce neuronal damage induced by permanent focal ischemia in rats, associated with regulation of HIF-1α protein and its target gene, HO-1 (71). Recently, a novel iron chelator/PHD inhibitor compound, TM6008 (Table 1) was shown to induce angiogenesis and to protect the brain against ischemia *in vivo* (213). The competitive PHD inhibitor DHB also was found to stabilize HIF-1α through its ability to bind iron (329) (Table 1). However, previous observation argued against this ability of DHB to bind iron in embryonic rat cortical neurons, while still demonstrating its capability of stabilizing HIF-1α and activating HIF-1–dependent genes (293). A recent report described that DHB and clioquinol protected dopaminergic neurons against MPTP-induced cell death and upregulated HIF-1α in C57Bl mice (168). DHB also elevated and maintained the levels of mRNAs and proteins of HIF-1α–dependent genes, HO-1 and Mn-SOD, in the presence of MPTP (168). Additionally, it was demonstrated that DHB and the iron-chelator SIH protected rat N27 SN DA-derived cells, cultured at oxygen-tension conditions, against MPP^+ toxicity (the oxidative product of MPTP) (168). These findings (Table 1) support the HIF-PHD inhibition and stabilization of HIF-1α as a novel relevant target for neuroprotective activity of iron chelators against oxidative and neurologic insults.

A. Multifunctional, iron-chelating neuroprotective drugs

Novel compounds comprising aromatic heterocyclic related to pyridine derivatives, such as 8-hydroxyquinolines, were previously developed as selective PHDs inhibitors (115, 330). The generation of 8-hydroxyquinolines is based on a

Table 1. Summary of HIF-1 System Regulation by Neuroprotective Compounds Possessing Iron-regulating Properties in Various Neuronal Cells and Animal Brains

Compound	*HIF system targets*	*Treatment protocol*	*References*
Desferrioxamine (DFO)	Inhibition of HIF-PHD and induction of HIF-1α	Rat primary cerebral cortical and hippocampal neurons; H19-7 hippocampal neuronal cells; neonatal mice; transient ischemia–reperfusion in neonatal rats, focal cerebral ischemic rat brain	(3, 6, 21, 25, 93, 108, 170, 212, 228, 241, 271, 292)
	HIF-1–target genes [*e.g.*, EPO; glycolytic enzymes ALDA, phosphoglycerate kinase 1 (PGK1), 6-phosphofructo-2-kinase/fructose-2, 6-bisphosphatase (PFKFB-1-4) and pyruvate kinase M; iNOS; TfR; $p21^{waf1/cip1}$; TH; VEGF; HO-1]	Rat primary cerebral cortical cells; hippocampal neurons from E16 CD1 mice; H19-7 hippocampal neuronal cells; human neural stem cells; rat brainstem; focal cerebral ischemic rat brain	(5, 66, 90, 148, 164, 292, 352)
Liposoluble 2,2′-dipyridyl	Regulation of HIF-1α protein and its target gene HO-1	Focal cerebral ischemic rat brain	(71)
	Regulation of its target gene HO-1	Focal cerebral ischemic rat brain	(71)
TM 6008	Blocking active site of PHD and FIH	Transient global ischemia of Mongolian gerbils, hypoxia-sensing transgenic rat strain	(213)
Ethyl 3,4 dimethylbenzoate (DHB)	Blocking active site of PHD, inhibition of HIF-PHD, and stabilization of HIF-1α protein	PC12 cells; rat primary cerebral cortical cells, rat N27 SN DA–derived cells, focal cerebral ischemic rat brain, MPTP-mouse model	(168, 292, 329)
	Induction of HIF-1–target genes (*e.g.*, GLUT-1; VEGF; ALDA and $p21^{waf1/cip1}$; HO-1; Mn-SOD)	PC12 cells; rat primary cerebral cortical cells, rat N27 SN DA–derived cells, MPTP mouse model	(168, 292, 329)
Clioquinol	Induction of HIF-1α	MPTP mouse model	(168)
M30 [5-(*N*-methyl-*N*-propargylaminomethyl)-8-hydroxyquinoline]; HLA20 [5-(4-propargylpiperazin-1-yl-methyl)-8-hydroxyquinol 1]	Induction of HIF-1α	Rat primary cortical neurons, motor neuron–like NSC-34 cells, adult mouse brain	(10, 159)
	HIF-1–target genes (*e.g.*, EPO; enolase 1; VEGF; p21; TH)	Rat primary cortical neurons, motor neuron–like NSC-34 cells, adult mouse brain	(10, 159)
Quercetin	Induced accumulation of HIF-1α	Murine brain endothelial cells	(342)
	HIF-1-target genes (*e.g.*, VEGF)	Murine brain endothelial cells	(342)
EGCG and related polyphenolic compounds (*e.g.*, ECG)	Induction of HIF-1α	SH-SY5Y cells	(103)
	HIF-1–target genes (*e.g.*, GLUT-1; VEGF; $p21^{waf1/cip1}$; iNOS; TfR)	SH-SY5Y cells; Rat nodose ganglion; ischemic rat brain	(310, 331–333)

recently described isoquinoline derivative that specifically inhibits PHDs. Co-crystallization with PHD2 revealed key interactions between this compound and the active site of the enzyme (200, 330), thereby serving as a basis for the design of neuroprotective compounds.

The neuroprotective multifunctional iron chelators 8-hydroxyquinolines derivatives, M30 and HLA20, were recently shown to regulate the HIF-PHD system (Table 1); M30 and HLA20 upregulated HIF-1α and significantly increased the levels of HIF-1-dependent neuroprotective genes, enolase-1, VEGF, and BDNF in motor neuron-like NSC-34 cells (159). In addition, these drugs upregulated HIF-1α and its dependent protective genes (*e.g.*, TfR, VEGF, BDNF, the glycolytic enzyme enolase 1, p21, and EPO) in rat primary embryonic cortical neurons (10). These gene products, which are regulated by HIF-1α, may mediate M30-induced neuroprotective effects (10, 159), because, for example, EPO was previously demonstrated to prevent neuronal cell death in a spectrum of different animal models (92, 93, 244), whereas the cyclin-dependent kinase inhibitor, p21, was shown to be involved in cell differentiation and cell-cycle arrest (206). In addition, prior studies suggested that VEGF confers neurogenesis and neuroprotective effects against cell death induced by various insults, such as OS, hypoxia, and glutamate excitoxicity (42, 166). This is consistent with our previous results (9, 11), showing that M30 induced neuronal differentiation, as manifested by cell-body elongation and stimulation of neurite outgrowth. In addition, recent data provided evidence that M30 induced mRNA expression levels of another important HIF-1–target gene, TH, used as a marker for neuronal recovery (10). Hypoxia was shown to induce TH gene transcription and stabilize TH mRNA in PC12 cells (66). Hypoxia induction of TH-gene transcription was reported to involve interaction of the c-Fos and Jun-B transcription factor with the AP1 site within the TH promoter (205, 217). Also, TH is regulated by pVHL and phosphorylated CREB (278).

It was reported that the PI3K-Akt signaling pathway activates a variety of signaling cascades with diverse outcomes, including neuronal survival and death (294, 365). PI3K-Akt activity also is intimately linked to HIF-1α regulation, not only by inducing HIF-1α translation in response to growth factors, but also through the regulation of HIF-1α protein degradation (140, 169, 284). Overexpression of a direct substrate of Akt, the glycogen synthase kinase (GSK)-3β, results in prolyl-hydroxylation and pVHL-independent HIF-1α ubiquitinylation and proteasomal degradation (80). Recent studies demonstrated that M30 significantly enhanced Akt and GSK-3β phosphorylation in embryonic cortical neurons and motor neuron–like NSC-34 cells (10, 159). Taken together, the neuroprotective and neurorestorative effects of the multifunctional iron-chelating drugs, demonstrated in several *in vitro* and *in vivo* neurodegenerative models, may correlate, at least in part, with their ability to activate the transcriptional activator, HIF-1α, and to upregulate the HIF-1 system.

B. Flavonoids

Flavonoids in nature, such as quercetin, galangin, and green-tea flavonoids, may constitute an additional promising therapeutic approach for treating various neurologic insults by the regulation of HIF-1 system. These compounds have been well characterized to posses antiinflammatory and antioxidant/prooxidant and divalent metal (iron and copper) chelating activities (102, 107, 158, 229) and have been shown to exert neuroprotection against a variety of neurotoxic insults (67, 74, 143, 191, 193, 336). The main pharmacological and iron-chelating properties of flavonoids have been accumulatively and comprehensively summarized (119, 235, 251). Extensive studies aimed at elucidating the molecular mechanisms and cell-signaling pathways participating in the neuroprotective action of flavonoids have revealed the regulation of a number of molecular targets, including the HIF/PHD pathway, by chelating cellular iron ions and/or through their radical-scavenging activities (137, 314, 361). Similar to hypoxia, natural flavonoids were demonstrated to induce intracellular accumulation of HIF-1α and subsquently the increase of VEGF secretion by inhibiting HIF-PHD in various cell lines and endothelial cells (229, 342). PI3K/Akt and MAPK/ERK signal-transduction pathways were found to be involved in the effect of flavonoids on HIF-1α (229). In support, the main constituent of the standardized dietary flavone, *Ginkgo biloba* (Ginkgoaceae) extract, EGb 761, was demonstrated to upregulate HIF-1α protein expression through the activation of the p42/p44 MAPK pathway, as well as significantly to protect hypoxic PC12 cells (181).

Recent studies in HeLa cells demonstrated that the flavonoids, baicalein, luteolin, fisetin, and quercetin, induced HIF-1α levels under normal oxygen pressure and revealed that their lipophilicity capability to bind intracellular iron efficiently was essential for HIF-1α induction (137, 317, 318). These reports suggest that the iron-chelating moieties in flavonoids (*e.g.*, quercetin; Fig. 6B; Table 1), the OH at position 3′ of the C ring and/or OH at positions 3′ and 4′ of the B ring, enabled PHD inhibition and consequently prevented HIF-1α ubiquitinylation and degradation.

Particular attention has been paid to studying the neuroprotective activity of flavonoid-type compounds, EGCG (128, 176, 178, 323). Several studies of short- and long-term EGCG administration, in different cellular and animal models of neurodegeneration, demonstrated various novel molecular targets, including, the MAPK/PKC and the PI-3K/Akt signaling pathways, survival genes and proteins associated with mitochondrial function, such as Bcl-2 family members (*e.g.*, Bcl-2, Bcl-W, and Bcl-xL), the binding protein 14-3-3, and heat-shock proteins (50, 54, 146, 155, 156, 177, 179, 216, 248–250, 332, 337). In addition, previous studies showed a potential neuroprotective effect of EGCG on the ganglionic neurons of the nodose ganglion (NG) in acute hypoxic rats (331) and against neuronal damage in various experimental models of hypoxia and cerebral ischemia (173, 182, 183, 309, 310, 331). These studies demonstrated that the favorable effects of EGCG are, in part, due to the modulation of NOS isoforms and to the preservation of mitochondrial-complex activity and integrity (331).

A recent proteomic study demonstrated the effect of EGCG on various proteins involved in the regulation of metabolic energy balance and neurite outgrowth, including ATP synthase H^+ transporting mitochondrial F1 complex β (333). These results suggest that the stimulation of cell energy by EGCG may be associated with its effect on mitochondrial function.

Moreover, EGCG and another member of the green-tea catechins, epicatechin-3-gallate (ECG) were previously shown to induce HIF-1α protein and mRNA expression levels of the HIF-1–target genes, GLUT-1, VEGF, and $p21^{waf1/cip1}$ (361). This effect was blocked by iron and ascorbate, indicating that these catechins may activate HIF-1α through iron chelation (314, 361). In accordance, a recent report described that ECG increased HIF-1α protein levels in cortical neurons subjected to ischemia induced by glucose deprivation (103). In support, in a progressive cell-death model of SH-SY5Y culture, EGCG was demonstrated to confer neurorescue effects and significantly to upregulate the expression of HIF-1α levels, as well as its target gene, associated with iron homeostasis, TfR (Table 1) (250, 333). Additionally, applying the same neurorescue paradigm, EGCG decreased mRNA transcript and protein levels of the β-subunit of PHD and the protein levels of the molecular chaperone member of the heat-shock protein (HSP)70 family, the immunoglobulin-heavy-chain–binding protein BiP, which is associated with HIF-1α regulation/degradation (250, 324, 332). Bip can replace the β-subunit of PHD in the enzyme tetramer structure (141); thus, the effect of EGCG on HIF-1α may be an outcome of downregulating the β-subunit of PHD and BiP. Moreover, EGCG decreased the expression level of HSP90-β in serum-deprived SH-SY5Y cells (332). Previous findings suggested that HSP90 inhibitors induced concentration-dependent regulation of the HIF-1 system, including VEGF expression–mediated HIF-1α stabilization (125, 149). Consequently, the regulatory effect of EGCG on the HIF-1 pathway under OS/hypoxic conditions may be associated with its iron-chelating activities, as well as with a suppressive effect on PHDs and neuronal regulation of HIF-1–target genes (333).

V. Conclusions and Future Perspectives

Although misregulation of the HIF/PHD pathway is only one component of a spectrum of reactions occurring in neurodegeneration, HIF-1 is a "master switch," being an important physiologic response mechanism, likely resulting in

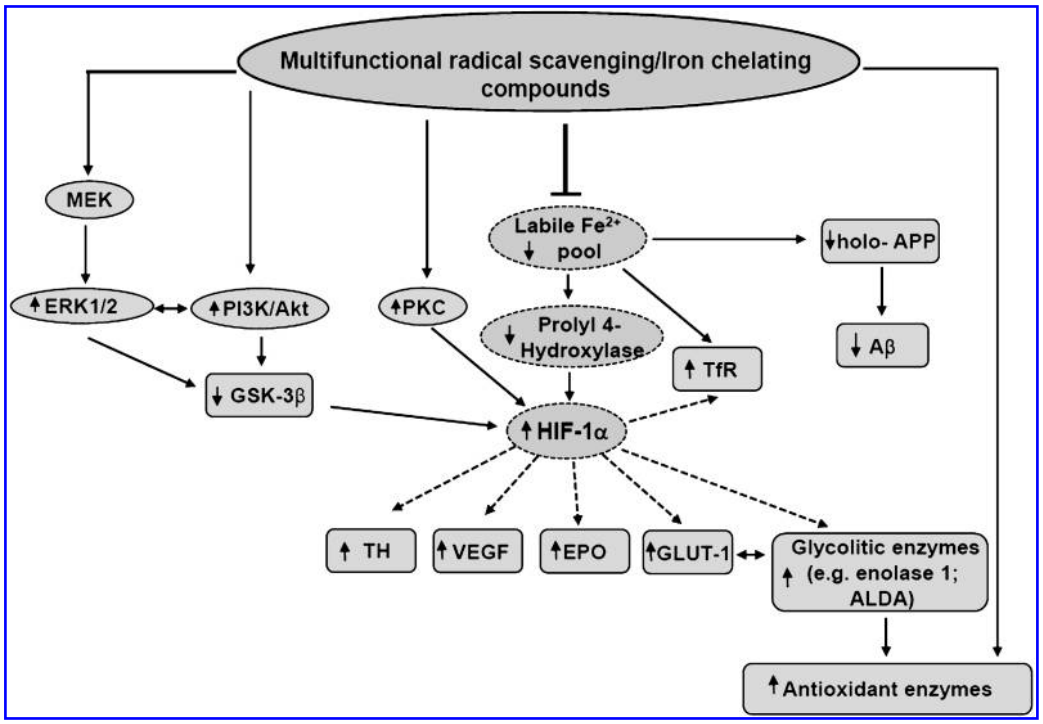

FIG. 11. Neuroprotective multifactorial effects of designed and natural iron chelators (*e.g.*, M30, HLA20, and flavonoids) associated with the HIF-1 system. A full explanation is discussed in the text.

several reproducible neuroprotective effects (29). Given the wide range and diversity of cellular functions regulated by the whole spectrum of HIF-1–target genes, it is suggested that this compensatory pathway mediated neuroprotection and is crucially involved in many physiologic processes within the brain (Fig. 11). Previous studies implicated reduced HIF-1α expression levels as a novel component in aging (a known risk for neurodegeneration) (261) and upregulation of EGLN1/PHD2 in human SN as a major biomarker in PD pathogenesis (100). Impaired activation of the HIF-1 system can alternatively occur by cellular disturbances of the proteasomal function, reported in many neurodegenerative diseases, such as PD and AD.

Iron-chelating stabilizers may exert their multifunctional neuroprotective and prosurvival effects by inducing the HIF-1α cell-signaling pathway and HIF-1–target genes, such as EPO and VEGF. Recent studies demonstrated that VEGF overexpression protected against nigral cell death induced in the MPTP model of PD (168). Thus, the novel notion and challenge underline the design of neuroprotective compounds, such as our brain-permeable multifunctional iron-chelating drug, M30, which would regulate the cellular redox state and activate HIF-1α signaling.

In addition, the analogous subunit of HIF-1α, HIF-2α, was suggested to play a crucial role in maintaining iron balance in the organism by directly regulating the transcription of the gene encoding the divalent metal transporter 1 (DMT1) (196). Specific deletion of HIF-2α led to a decrease in serum and liver iron levels and a marked decrease in liver hepcidin expression, indicating the involvement of an induced systemic response to counteract iron deficiency (196). HIF-2α expression, not like that of HIF-1α, which is constitutively expressed, appears to be restricted to certain brain areas, including the cortex, as well as the hippocampus and cerebellum (341). Additionally, recent study suggested that a target for iron chelator–mediated neuroprotection is PHD1-upregulated HIF-2, which is an iron, oxygen, and 2-oxoglutarate–dependent dioxygenase (292). This suggests that monitoring HIF-2α levels in the brain could benefit patients with iron-related neurodegenerative disorders, considering that its activation may allow iron mobilization, whereas its reduction, by iron-chelator drugs, favors decreased iron absorption.

However, the more-precise impact regarding the specific mechanisms by which iron chelators regulate HIF-1/2 systems must be further investigated. Future transgenic animal–model studies, selectively modulating HIF-1/2 expression and function in the brain, will clarify the importance of this pathway in the neuroprotective effect of iron-chelating drugs, broaden the current therapeutic approaches for the treatment of neurologic disorders, and overall, will open a new window for future drug development. Because iron chelation has been suggested to be a therapeutic treatment for disorders such as PD, AD, and ALS, understanding the exact mechanisms by which iron acts to cause neurodegeneration and how the brain would be affected by iron chelation could potentially give us novel insights into new therapies directed toward preventing or slowing neuronal death.

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Abbreviations Used

5′UTR = 5′-untranslated region
6-OHDA = 6-hydroxydopamine
Aβ = β-amyloid peptide
AD = Alzheimer's disease
ADAM10 = metallopeptidase 10
ADM = adrenomedullin
ALD = aldolase
ALS = amyotrophic lateral sclerosis
ARNT = aryl hydrocarbon receptor translocator
BBB = blood–brain barrier
BDNF = brain-derived neurotrophic factor
ChAT = choline acetyltransferase
ChE = cholinesterase
$CoCl_2$ = cobalt chloride
CREB = cAMP response elements–binding protein
C-TAD = C-terminal transactivation
CXCR = stromal-derived factor
DA = dopamine
DFO = desferrioxamine
DMOG = dimethyl-oxalyl-glycine
EGCG = green tea (-)-epigallocatechin-3-gallate
EGLN = egg-laying–defective nine
EPO = erythropoietin
(Fe^{2+}) = iron
G6PD = glucose 6-phosphate dehydrogenase
GAP-3 = growth-associated protein 43
GDNF = glial cell line–derived neurotrophic factor
GLUT-1 = glucose transporter 1
GP = glutathione peroxidase
GSH = glutathione
GSK = glycogen synthase kinase
HFE = hemochromatosis
HI = hypoxia–ischemia
HIF-1α = hypoxia-inducible factor-1α
HO-1 = heme oxygenase
H_2O_2 = hydrogen peroxide
HREs = hypoxia-response elements
HSP = heat-shock protein
IGF-1 = insulin-like growth factor 1
iNOS = nitric oxide synthase
IRPs = iron-regulatory proteins
L-DOPA = L-dihydroxyphenylalanine
MAPK = mitogen-activated protein kinase
MPTP = *N*-methyl-4-phenyl-1,2,3,6-tertahydropyridine
MT = metallothioneines
NADPH = nicotinamide adenine dinucleotide phosphate
NG = nodose ganglion
NGF = nerve growth factor
NMDA = *N*-methyl-D-aspartic acid
NOS = nitric oxide synthase
ODDD = oxygen-dependent degradation domain
OGF = opioid growth factor
OS = oxidative stress
PD = Parkinson's disease
PFKFB-1-4 = 6-phosphofructo-2-kinase/fructose-2, 6-bisphosphatase
PGK1 = phosphoglycerate kinase 1
PHD = prolyl-4-hydroxylase
PI3K = phosphatidylinositol-3-kinase
PKC = protein kinase C
PT = permeability transition
pVHL = von Hippel-Lindau
ROS = reactive oxygen species
SIN-1 = 3-morpholino sydnonimine
SNpc = substantia nigra pars compacta
SOD = superoxide dismutase
SSRIs = selective serotonin-reuptake inhibitors
TfR = transferrin receptor
TGF-β = transforming growth factor-β
TH = tyrosine hydroxylase
TNF-α = tumor necrosis factor-α
Trk = tyrosine kinase receptor
VEGF = vascular endothelial growth factor

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