Antioxidant and cholinergic neuroprotective mechanisms in experimental parkinsonism

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Summary

Many instruments have been employed in recent years in order to quantify the posture and motion of the head in normal and pathological subjects. Evaluations of this type present many difficulties related to the influence of individual and external factors and to the accuracy of the system used. In patients with cervical dystonia (CD) the only rating scales currently used are semi-quantitative and subjective. More precise information on disease severity and response to therapeutic or improvement and the restriction of motion before treatment.

KEY WORDS: Antioxidants, basal ganglia, dopamine neuroprotection, nicotinic receptors, Parkinson's disease.

Introduction

Since the main biochemical manifestation of Parkinson's disease (PD) is a pronounced deficiency of striatal and nigral dopamine (DA), the pharmacological approach for treating PD has been based on the restoration of dopaminergic neurotransmission, by administration of DA precursors (L-Dopa), DA agonists, or both (1,2). However, these therapies present serious disadvantages in terms of their side effects and their loss of efficacy, observed in long-term treatments (3,4). Because of these clinical limitations, new and more efficient therapeutic alternatives have been developed. Although the aetiology of PD is not precisely known, understanding of the mechanisms involved in parkinsonian neuronal death is increasing. At present, it is generally accepted that an array of factors (genetic, toxic, vascular, oxidative, etc.) ultimately lead to the progressive loss of dopaminergic neurons. In addition, there is a growing body of data indicating that oxidative stress plays – as a cause, a consequence or both – a pivotal role in the development of neurodegenerative disorders (5-7).

The term oxidative stress refers to the cytological consequences of a mismatch between the production of reactive oxygen species (ROS) and the ability of cells to protect themselves against ROS (Fig. 1, see over) (7). These imbalances in oxidative metabolism can be considered a final common pathway in a great variety of cellular insults and pathologies. Because PD is a chronic disease characterised by progressive clinical development and concurrent neuronal death, it can be hypothesised that therapeutic interventions designed to tackle oxidative stress could also protect SN neurons, acquiring clinical importance as adjuvant therapies. However, the failure of α-tocopherol in the large multicentre clinical DATATO P trial (8) has prompted experimental analysis of alternative antioxidant strategies. In the use of antioxidant therapies, oxidative stress is commonly regarded as a consequence of – as yet ill-defined – pathological processes, and the therapeutic strategies are limited to the direct neutralisation of free radical concentrations (use of scavengers) or the promotion of enzymatic antioxidant defences. An alternative approach is the utilisation of compounds capable of supporting neuronal survival by enhancing cellular defences and/or preventing neuronal mechanisms of death (i.e., apoptosis). As an example, a variety of nerve growth factors, nicotinic drugs, antiapoptotic effectors, etc., have been shown to protect neurons in several experimental models (8).

We have examined these questions, testing the neuroprotective capacity of naturally occurring antioxidants with a predominant scavenger profile. An important objective of an antioxidant would be to neutralise, by scavenging activity, the production of the hydroxyl radical (OH•) (Fig. 1) (9-12). Given that the incidence of PD is lower in smokers – this is an established epidemiological fact – we have also analysed the ability of neuronal nicotinic acetylcholine receptors (nAChRs) to stimulate cellular defences and counteract the progressive dopaminergic loss.

In both cases we utilised the classical 6-hydroxydopamine (6-OHDA) model of PD (13).

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Substantia nigra, oxidative stress and 6-OHDA

The brain is regarded as vulnerable to oxidative stress, and one of the reasons for this is the considerable...
amount of oxygen it requires to maintain its activity. The substantia nigra (SN) is thought to be particularly exposed due to the generation of ROS in the course of DA metabolism and autooxidation (14). This situation is worsened by the increased iron content and relatively low levels of catalase, ferritin and glutathione in the parkinsonian SN (15-17). Neurons in the SN might, as a cell population, be considered particularly sensitive to imbalances in ROS and oxidative stress, with a marked production of ROS expected after any oxidative insult. However, recent experimental evidence is showing that the brain has important antioxidant defences that can cope with marked oxidative insults. In particular, Piantadini et al. (18) reported no increase in hippocampal OH• production after thirty minutes of hypoxia and Pearigen et al. (19) demonstrated that profound hypoxia (pO₂ = 20 mm/Hg) is well tolerated by the brain. In accordance with these observations, a single episode of hypoxia-reoxygenation, in vivo, failed to increase OH• production (measured as generation of 2,3 dihydroxybenzoic acid, 2,3 DHBA) (20) (Fig. 2) or lipid peroxidation (measured as tissue concentrations of TBARS) in the striatum or SN. Any hypoxic process that compromises the energy supply to the central nervous system (CNS) elicits a cascade of events involving ROS production that may ultimately end in neuronal death and brain injury (21-23). The lack of changes in OH• levels after hypoxia-reoxygenation might be an indication of the ability of endogenous antioxidant defences to counterbalance an increased generation of ROS. Since blood circulation is maintained during hypoxia, the transportation of antioxidants, e.g., ascorbate, from the blood to the cell would not be altered and may even be increased, reaching concentrations critical for counteracting oxidative stress.

When studying the biochemistry of DA metabolism, we found that hypoxia-reoxygenation provokes an increase in DA concentrations and a decrease in DA metabolites (dihydroxyphenyl acetic acid, DOPAC, and homovanillic acid, HVA) in the SN (Fig. 3, unpublished data). The SN would therefore appear to respond to an oxidative insult like acute hypoxia-reoxygenation in an efficient manner, decreasing DA utilisation, as discussed above, and controlling ROS production, most likely by maximal utilisation of antioxidant defences. This adaptive equilibrium is, nevertheless, fragile, as can be seen when hypoxia-reoxygenation is combined with a mild 6-OHDA insult. The 6-OHDA model of SN injury has been utilised for many years as a classical experimental model of parkin-
sonism. Unilateral stereotaxic injection of 6-OHDA into the SN of the rat leads to a progressive and massive death of the dopaminergic nerve cells on the ipsilateral side and to a corresponding depletion of DA in the corpus striatum (24). 6-OHDA is thought to produce nigrostriatal dopaminergic lesions through the production of ROS and the generation of oxidative stress (25, 26). However, no significant changes in OH$^•$ production, assessed through the detection of 2,3 DHBA, are observed in the SN after a 6-OHDA insult (Fig. 2).

When hypoxia and 6-OHDA insults are combined by adding an episode of 60 min. hypoxia after the intranigral injection of 6-OHDA, OH$^•$ production increases significantly above basal levels, and is accompanied by a worsening of the DA loss in the striatum (20) (Fig. 4). If we consider that morphologically demonstrated loss of neurons in the pars compacta of the SN significantly correlates with reduction of DA in the striatum (1, 27), the increased OH$^•$ production would correspond to an increased neurotoxicity of 6-OHDA after the addition of the hypoxia. When hypoxia is performed early after the intra-nigral injection of 6-OHDA the consequence would be significant increase in OH$^•$ production, accompanied by long-term increased neurodegeneration in the SN. These experimental data clearly support the importance of studying the use of antioxidant scavengers as neuroprotectors.

Considering the putative role of DA metabolism in the development of oxidative stress, it is interesting to compare DA utilisation in the SN after the experimental conditions described above: hypoxia alone decreased DA turnover and did not provoke a significant increase in OH$^•$. However, when it was combined with 6-OHDA we observed an augmented DA turnover together with a significant increase in OH$^•$ production (20).

SN neurons have strong antioxidant defences that allow them to cope with oxidative episodes. But increased intensity of the insult, its repetition or its summation with other oxidative circumstances would overcome these antioxidative barriers. The 6-OHDA model thus provides an unstable oxidative scenario that probably resembles the oxidative process in the SN in PD, and constitutes an adequate experimental design to assess the neuroprotective potential of different molecules.

Natural antioxidants

Two decades ago, semi-synthetic apomorphine was used extensively in clinical pharmacology for the treatment of PD, mainly on the basis of its dopaminergic agonism (28, 29). Apomorphine in particular, belongs to a family of natural antioxidants, known as aporphines. Boldine, a natural aporphine alkaloid, has recently been
shown to have protective effects, on isolated hepatocytes and red blood cells, against free-radical insults (30,31). Taking into account these antecedents, natural aporphines could represent important alternatives for the management of early oxidative stress in experimental parkinsonism (Fig. 5).

Boldine can be identified in the nervous tissue five minutes after systemic administration. However, its presence in the brain did not affect OH• production during a hypoxic episode, nor did it counteract the dopamine loss provoked by 6-OHDA. Paradoxically, boldine actually exacerbated the DA decrease after 6-OHDA (Fig. 6, unpublished data).

Why doesn't a potent natural antioxidant like boldine protect SN neurons, in vivo, in an early stage of an oxidative insult? A plausible explanation is that in the 6-OHDA model of brain lesion, the scavenging properties of boldine would be undermined by its capacity to function as a dopaminergic antagonist (32). In this regard, DA antagonism increases DA utilisation and can therefore increase oxidative stress, actions that would ultimately counteract the antioxidant protection afforded by its free radical absorption.

The results obtained after boldine treatments appear to show that if scavenging properties are accompanied by a pharmacological action that enhances DA metabolism, the value of the antioxidant capacity per se could, in efforts to prevent the neuronal loss in the SN after 6-OHDA, be only partial.

At present, we are in the initial stages of characterising other natural aporphines, with more adequate pharmacological profiles. In this regard, pukateine, ([R]-11-hydroxy-1,2-methylenedioxyaporphine, (Fig. 5)], an aporphine alkaloid present in the bark of the pukatea tree (Laurelia novae-zelandiae), has given promising results. Showing an agonist-like interaction with DA receptors, and an only moderate increase in extracellular DA, pukateine shows a potent antioxidant activity (32) that makes it a plausible alternative for testing the putative neuroprotective actions of natural aporphines in vivo.

Considering that antioxidant properties per se may not be sufficient for the development of an efficient neuroprotective drug, we further investigated the neuroprotective action of antioxidant molecules also capable of activating enzymatic responses.

Quercetin is a natural flavonoid widely present in nature. Its three-ring flavonoid structure provides it with a marked scavenger potency, which is greater than that of structurally analogous molecules like rutin, kaempherol, etc. (33). In addition, quercetin inhibits xanthine-oxidase and PI-4 and PI-5 kinases, prevents platelet aggregation and has antiviral and carcinostatic properties (34-36). Compared with boldine, quercetin is a potent scavenger with additional antioxidant activity: for example it inhibits xanthine-oxidases and kinases that would decrease ROS production (37). Nevertheless, quercetin does not reverse the striatal dopaminergic loss provoked by intranigral injection of 6-OHDA. Once again, these results would seem to indicate that molecules with a dominant scavenger activity are not effective neuroprotective agents in the 6-OHDA model of experimental parkinsonism.

Well known for its marked circadian rhythm and neuroendocrine-like properties, melatonin, one of the end products of tryptophan, has been associated with direct and indirect antioxidant properties (38). A ubiquitous antioxidant that increases levels of superoxide dismutase, melatonin plays a significant role in removing \( \text{H}_2\text{O}_2 \) from cells by modulating the activity of glutathione peroxidase. It can reduce NO production by restricting the activation of nitric oxide synthase, while also acting as an OH• scavenger (39). In addition, melatonin reduces the toxic effects of kainic acid and ischaemia, as well as the cytotoxicity of 6-OHDA in cell cultures. The marked reduction of melatonin production with age has prompted some authors to postulate a role for this molecule in neurodegenerative disorders (38,39). Therefore, melatonin is an ideal candidate for studying protective alternatives in the 6-OHDA model of neural degeneration. The systemic administration of melatonin, thirty minutes before an intranigral injection of 6-OHDA,
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significantly prevented the loss of DA in the striatum (unpublished data). As shown in figure 6, melatonin presented an antioxidant scavenger profile with an IC50 value more than one order of magnitude higher than those of boldine and quercetin, in vitro lipoperoxidation assays. However, melatonin’s indirect antioxidant actions would make it a more effective neuroprotective molecule.

Nicotinic acetylcholine receptors and neuroprotection

The description by several authors of a weak neuroprotective effect of smoking in PD has lent weight to the hypothesis of a protective role of nicotine on CNS neurons (40). In apparent agreement with this fact, numerous in vitro reports have confirmed the neuroprotection conferred by nicotine against a variety of toxic insults, such as excitotoxicity (41-45), beta-amyloid toxicity (46), and serum deprivation (47). Blockade of the neuroprotective effects by nAChR antagonists provides further evidence in support of the hypothesis of specific mediation by particular subtypes of nAChR (48,49).

In vivo studies, however, have shown some discrepancies over the question of nicotine neuroprotection. While continuous nicotine infusion has been demonstrated to protect against neuronal loss provoked by DA pathway hemitransection (50), striatal depletion of DA provoked by SN 5-OHDA injection was unaltered by the same treatment (51). Systemic application in vivo of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) resulted in a significant reduction of striatal DA that was not reversed by nicotine (52,53), while in another study nicotine showed protective effects against diethyldithiocarbamate enhancement of MPTP lesion (54). In addition, in the MPTP model of experimental Parkinsonism, nicotine has also produced an enhancement of the observed neurotoxicity (55,56).

A possible explanation for these apparently contradictory results could lie in the great variety of experimental conditions described in the literature, which are characterised by marked differences in the schedule and route of nicotine administration. Accordingly, chronic treatments did not show protective effects in vivo (51-53) while acute intermittent administration did (54,55). However, Janson et al. (50) showed nicotine protection after chronic and continuous treatment in a hemitransection model of neuronal death.

Because nicotine has negligible antioxidant scavenger capacity in in vitro models, any neuroprotective effect observed after an oxidative lesion provoked by 6-OHDA could only be attributed to its general capacity to stimulate antioxidant defences or to activate intracellular pathways involved in preventing neuronal death. For these reasons, we studied the putative neuroprotective effects of nicotine in the 6-OHDA model of experimental Parkinsonism, assessing whether the timing and schedule of nicotine administration, as well as the extent of the lesion, are factors that could effectively influence the neuroprotective profile (13).

Nicotine was applied subcutaneously both 4 hours before and 20, 44 and 68 hours after 6-OHDA injection, significantly prevented striatal DA loss and increased DA turnover. However, when administration took place only before (4 hours before) or after (20, 44 and 68 hours after) 6-OHDA injection, it did not reverse the DA loss. These findings serve to underline the importance of treatment schedule. Chlorisondamine, a long-lasting nicotinic acetylcholine receptor antagonist (57,58) blocked the effect of nicotine on DA concentrations and DA turnover, confirming the role of nAChRs in the mediation of neuroprotection.

In view of the striking correspondence between the pattern of the protection given by nicotine and the growth factor production peaks provoked by nicotine treatment in previous studies (59-61), it is possible to assume that nAChR protection occurs through an increased release of trophic factors, which would subsequently activate intracellular cascades leading to neuronal survival. In this regard, it has previously been shown that growth factors can protect neurons against a variety of toxic insults, such as excitotoxicity and/or ischaemia-reperfusion (61). It is interesting to note that chronic administration of nicotine, most probably provoking desensitisation of nicotinic receptors, did not show any protective effects (62,63).

Although an indirect protection by nicotine through increased release and/or production of trophic factors is a likely mechanism, it is also possible that nAChR activation per se could trigger intracellular cascades, leading to the prevention of neuronal loss. In this regard, nAChRs have been shown to promote the direct activation of MAP kinase (64), a pathway commonly described as an intracellular transducer of nerve growth factor receptors. Additionally, a report by Heusch et al. (65) further substantiated the possibility of a direct protective action of nAChRs, when showing that nicotine, also through a MAP-kinase dependent pathway, can promote an increase in the anti-apoptotic effector bcl-2.

Concluding remarks

The cholinergic experiments described above add weight to the central hypothesis we are advancing, i.e., that scavenger activity per se does not confer full protection against oxidative stress in vivo, in experimental Parkinsonism. The results presented show that strong scavengers like boldine or quercetin do not protect against DA loss, whereas nicotine, a molecule without particular antioxidant activity, showed significant protective properties when applied in an intermittent manner. In the same way, melatonin strongly prevented neurodegeneration of DA neurons while presenting only poor anti-lipoperoxidative properties.

It is therefore important to remark that the direct scavenging action of the different molecules assayed was not directly correlated with an increased efficiency in neuroprotection. On the contrary, the weakest performers in the lipoperoxidation assay were the stronger inhibitors of DA loss (Fig.s 6 and 7, unpublished data). As a whole, the results obtained lend further support to the idea that direct antioxidant properties should not be the main and only determinants in the search for and development of neuroprotective strategies in degenerative disorders like Parkinson’s disease.
Fig. 7 - IC50 values (concentration inhibiting peroxidation by 50%, measured by inhibition of TBARS formation in brain tissue homogenates), following quercetin, boldine, pukateine and metelatonin. IC50 values were calculated from the concentration-response curve.

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