Biomedical Sciences
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Serum protein halogenation and nitrosylation: trait of maintained overstimulation of blood phagocytes in sporadic Parkinson's disease

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Abstract

Introduction

Halogenative and nitrosative stress are two types of oxidative stress that have been proposed as pathogenic mechanisms in Parkinson's disease (PD). They can be caused by overstimulation of phagocytes. This hypothesis discusses serum protein halogenation and nitrosylation as traits of maintained overstimulation of blood phagocytes in sporadic Parkinson's disease.

Hypothesis

I hypothesize that maintained phagocyte overstimulation leads to both halogenative and nitrosative stress in PD, which are present in the serum and cerebrospinal fluid of patients. These types of oxidative stress could modify proteins related to the pathogenesis of PD.

Evaluation of hypothesis

It has been detected that the presence of halogenative stress in the serum and, to a lesser extent, cerebrospinal fluid of Parkinsonian patients leads to excess of advanced oxidized protein products. On the other hand, nitrosative stress is also present in serum and cerebrospinal fluid of patients with early PD, characterized by the selective increase of 3-nitrotyrosine proteins other than nitroalbumin and free 3-nitrotyrosine. Nitrosylation stress accompanies modification of the sites of nitrosylation of α-synuclein in these patients, characterized by dominant nitrosylation of tyrosine 125/136 residues.

Conclusion

Since metabolism of advanced oxidized protein products and 3-nitrotyrosine proteins has been associated with phagocytic overstimulation, this pathological alteration could play a pathogenic role in sporadic PD. Our observations also lead to the hypothesis that serum level of advanced oxidized protein products is a prognostic marker for PD duration, and these oxidized proteins could participate in neuroinflammation. Besides, the evaluation of nitrosative stress through enhanced levels of 3-nitrotyrosine proteins in serum and cerebrospinal fluid without changes in nitroalbumin, together with the profile of tyrosine nitrosylation of serum α-synuclein characterized by dominant nitrosylation of Tyr125/136, could serve for the diagnosis of sporadic PD. Nitro-α-synuclein is a main component of Lewy bodies, hallmarks of the disease, and serum nitro-α-synuclein could represent a pathogenic factor in PD.

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Introduction

Oxidative stress is defined as an imbalance between the production of reactive oxygen and nitrogen species (ROS/RNS) and antioxidant mechanisms, and it is considered as an important pathogenic mechanism in Parkinson's disease (PD)[1–4]. Halogenation is a type of oxidative stress induced by phagocytic overstimulation, leading to excess of several molecules such as advanced oxidized protein products (AOPPs). These products originate as a result of the action of free radicals such as chloramines and hypochlorous acid (HOCl) on proteins, and they are augmented in several diseases[5]. This halogenative process could also modify free amines such as tyrosine, leading to the formation of free 3-chlorotyrosine, as shown in Figure 1.

Another type of oxidative stress is nitrosylation, where oxidative modifications of proteins are secondary to excess of nitric oxide. Nitrosylation of tyrosine leading to 3-nitrotyrosine proteins or free 3-nitrotyrosine is the most prominent change, and this type of oxidative stress has been associated with human pathologies[6], and it is recognized as a salient feature of diverse α-synucleinopathies such as PD and Lewy body dementia[7]. In PD, protein tyrosine nitrosylation has been demonstrated to represent a pathogenic mechanism, and nitrosylated tyrosine residues of diverse proteins such as neurofilaments or α-synuclein are detected in the brain proteinaceous aggregates or Lewy bodies, hallmarks of the disease[8]. Moreover, nitrating agents generated by dopamine are known to promote α-synuclein aggregation[9], a critical step for the formation of Lewy bodies. Biochemically, nitrosylated proteins and amines can be formed through different pathways (Figure 1). The formation of peroxynitrite (ONOO–) through nitric oxide and superoxide anion is the main pathway[10]. However, other nitrating pathways related to phagocyte overstimulation exist. For instance, myeloperoxidase (MPO) activity and nitric oxide excess from macrophages or brain microglia yield nitrosylated proteins and nitrotyrosines[11]. This hypothesis discusses if protein halogenation

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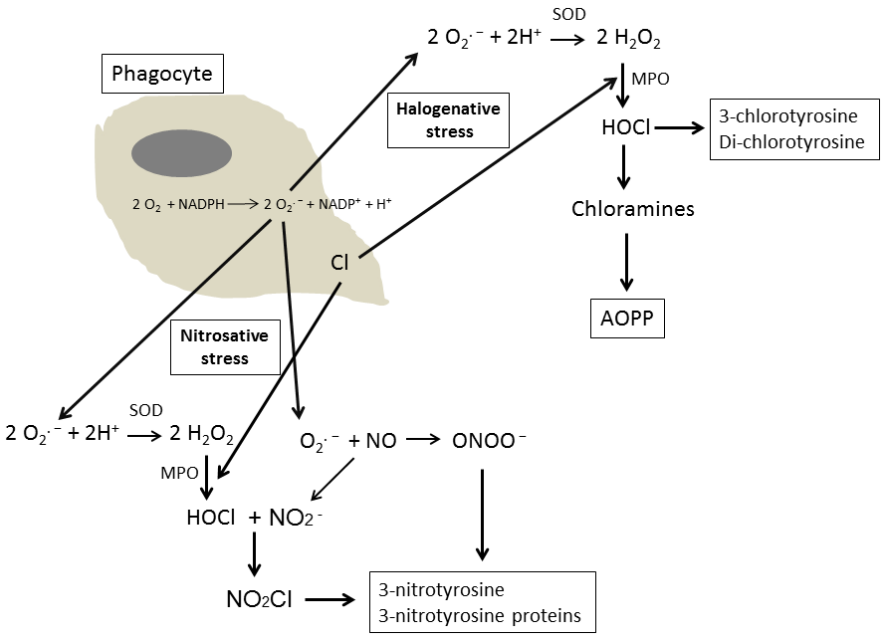

Figure 1: Main metabolic pathways of halogenative and nitrosative stress related to phagocyte overstimulation. Halogenated proteins or AOPPs, and free 3- and di-chlorotyrosines, originate as a result of the action of free radicals such as chloramines and hypochlorous acid (HOCl). These radicals are formed from hydrogen peroxide (H_2O_2) due to the release of superoxide anion (O_2^-) from phagocytes and the action of superoxide dismutase. Nitrosative stress leading to nitrosylated proteins and nitrotyrosines is caused by two main pathways. First, peroxynitrite (ONOO–) is formed through nitric oxide (NO) and superoxide anion released by phagocytes. Second, MPO activity from phagocytes yields hypochlorous acid that reacts with nitrogen dioxide (NO_2^-) derived from nitric oxide and superoxide anion, and nitryl chloride (NO_2Cl) is formed. Both peroxynitrite and nitryl chloride can induce the formation of 3-nitrotyrosine proteins and free nitrotyrosines. NADPH, nicotinamide adenine dinucleotide phosphate; MPO, myeloperoxidase; SOD, superoxide dismutase; H+, hydrogen; AOPP, advanced oxidized protein products.

and nitrosylation are caused by overstimulation of phagocytes in PD.

Hypothesis

I hypothesize that both halogenative and nitrosative stress are present in serum and cerebrospinal fluid (CSF) of PD patients, and both types of oxidative stress would modify proteins which could be related to the pathogenesis of PD[12,13].

Evaluation of hypothesis

Informed consent forms under a protocol approved by the University of Seville and Macarena Hospital internal ethics and scientific boards were obtained from all the subjects, and the subjects' consent was obtained according to the Declaration of Helsinki (BMJ 1991;302:1194). First, AOPP levels were quantified through ELISA, and they were found to be higher in serum and CSF of PD patients relative to control, but only serum AOPP values were significantly different. Taking into account Hoehn–Yahr stages of the disease, AOPP levels were progressively reduced in serum, and levels in advanced or Hoehn–Yahr stage 3 and 4 patients are similar to controls. Since halogenative oxidation can also yield halogenated amines, the presence of 3-chlorotyrosine was evaluated in the serum and CSF from PD patients with mass spectrometry. 3-Chlorotyrosine was not detected in any fluid. To further analyse AOPP properties, simple linear regression analyses between AOPP levels and clinical characteristics were carried out. In PD patients, serum AOPP levels found to be related to Hoehn–Yahr stage and to levodopa dosage. The higher Hoehn–Yahr stage or levodopa dose, the lower serum AOPP levels. Besides, considering individual duration of disease and AOPP levels in stage 2/3 patients, a significant regression was observed between patients and serum AOPP levels[12]. It seems that serum AOPP levels predict how large the disease is in this group of patients.

Second, the presence of nitrosative stress in the blood and CSF of PD patients was evaluated through ELISA of 3-nitrotyrosine proteins. Serum 3-nitrotyrosine protein levels were significantly enhanced in early-PD patients (Hoehn–Yahr stages 1 and 2). Interestingly, other markers of nitrosylation such as serum nitroalbumin or free 3-nitrotyrosine were not affected. On the other hand, CSF 3-nitrotyrosine protein levels were also enhanced in patients and, taking into account Hoehn–Yahr stages, this effect was detected in early-PD patients again. Besides, neither nitroalbumin nor free 3-nitrotyrosine was affected in CSF of patients. These findings suggested that nitrosylation was quite selective, and I decided to discern which proteins are affected by nitrosylation stress. By means of western blotting and one-step filtered serum (Amicon 50K filters), a 3-nitrotyrosine protein band of approx. 56 kDa in serum from patients and controls was detected, corresponding to 3-nitrotyrosine α-synuclein, because the same signal was detected after both anti-3-nitrotyrosine and anti-α-synuclein antibodies. A approx. 56 kDa band is characteristic of tetrameric

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α-synuclein, the physiological form of endogenous α-synuclein[14]. When levels of total α-synuclein in serum through ELISA were measured, no differences were found. Regarding CSF, α-synuclein levels were found to be significantly lower in patients relative to controls, as already reported by others[15], but a nitrosylated band of approx. 56 kDa was not detected. Hence α-synuclein is present in CSF of patients with PD at lower values than normal, but it seems not to be nitrosylated[13].

Consequence of hypothesis

To further characterize the effects of nitrosative stress on serum nitro-α-synuclein (N-αSyn), and taking into account that tyrosine residues of N-αSyn can be differentially nitrosylated leading to different functional effects[16–21], the sites of tyrosine nitrosylation of the molecule were evaluated. Changes in the nitrosylation of tyrosine residues located at the carboxyl-terminus of the molecule (tyrosines at sites 125/136) and at the amine-terminus (tyrosine residue at position 39) were observed. Thus intensity of nitrosylation at Tyr125/136 sites was enhanced in Hoehn–Yahr stage 1 and 2 patients, and nitrosylation of the tyrosine 39 site was found to be reduced in stage 1 patients relative to controls. Representative blot bands of nitrosylation at Tyr125/136 residues of N-αSyn on early stage 1 patients are shown in Figure 2. On measuring the ratio defined as the intensity of N-αSyn-Tyr125/136 band between intensity of N-αSyn-Tyr39 band, it was found to be significantly elevated in patients with early PD versus advanced patients and controls, as summarized in Table 1. Thus all patients at Hoehn–Yahr stage 1, and 90% of patients at stage 2 showed a ratio higher than 1.6, while this ratio was ever observed to be lower than 1.6 in control subjects and advanced patients. This value could beconsidered as a limit betweenpatients with early PD and controls[13].

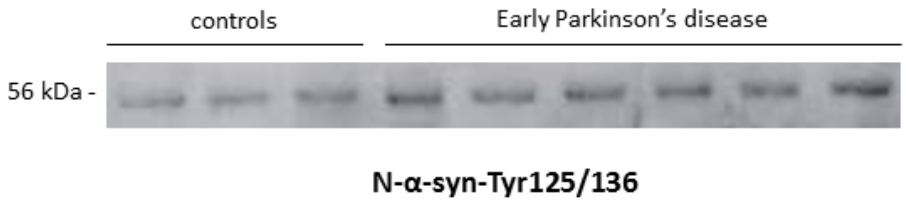

Figure 2: Representative blot bands of patients with early Parkinson's disease (Hoehn–Yahr stage 1 group) and controls, after N-αSyn Tyr125/136 monoclonal antibody. The intensity of blot bands is observed to be enhanced in patients. N-αSyn Tyr125/136, nitrosylated αSyn with nitrosylation of tyrosine residues 125/136.

Table 1 Ratio of intensity of N-αSyn-Tyr125/136 blot band between intensity of N-αSyn-Tyr39 band in early and advanced patients with Parkinson's disease (PD) and controls

	Controls	Early PD	Advanced PD
Ratio (mean ± SEM)	0.95 ± 0.1	3.2 ± 0.4**	0.92 ± 0.2

** $P < 0.01$ versus controls and advanced patients (Student's *t* test)

Discussion

Our studies show that there are indicators of halogenative oxidation stress in serum and, to a lesser extent, cerebrospinal fluid of PD, characterized by enhanced levels of AOPPs without changes in free 3-chlorotyrosine. The findings also indicate that serum and CSF AOPP levels are progressively reduced over time, and levodopa treatment contributes to these changes.

Metabolism of AOPPs has been associated to several diseases previously[5], where blood phagocytes are overstimulated and the formation of HOCl and chloramines from chloride and released hydrogen peroxide is enhanced. MPO catalyses this process and, as a consequence, AOPP are formed due to oxidative stress. Since AOPPs can conjugate with human seroalbumin (HSA) giving AOPP-HSA conjugates, inflammatory mediators, it can be hypothesized that AOPPs could be linked to neuroinflammation, a process whose pathogenic role in PD is widely accepted[22]. Of note is that low AOPP levels would predict a larger duration of PD. It seems that AOPP level in serum, not CSF, is a prognostic marker of duration and severity of disease, likely because it is a factor contributing to neuroinflammation or that blood halogenated proteins contribute to the cascade of events leading to neuronal degeneration.

Our findings also indicate that, in serum and CSF of early-PD patients, there are signs of nitrosative stress affecting proteins. This nitrosylation process was found to be quite selective because 3-nitrotyrosine proteins but not nitroalbumin or nitrosylated amines such as 3-nitrotyrosine were affected. In this context, the selectivity of tyrosine nitration in many diseases has been highlighted by several authors[6,7], and it has been explained by enzymatic catalysis and proximity to sources of nitric oxide synthesis[23]. This selectivity was corroborated after detecting a specific change in N-αSyn, one of the 3-nitrotyrosine proteins. This protein was not found to be enhanced in the serum, and CSF was devoid of its presence, but the profile of nitrosylation of tyrosine residues of serum N-αSyn was selectively changed, in early or Hoehn–Yahr 1

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and 2 patients. This selective change was characterized by dominant nitrosylation of Tyr125/136 residues and low nitrosylation of Tyr39 site. In other words, the carboxyl-terminus tyrosine sites of the molecule show higher nitrosylation than the amine-terminus one. The ratio between blot intensity of both parameters (N-αSyn-Tyr125/136:N-αSyn-Tyr39 ratio) was significantly higher in patients with early PD relative to controls and advanced patients. A ratio higher than 1.6 was established as a potential biomarker of early PD. These nitrosative effects were progressively reduced over time, for unknown reasons.

Such a selective change in tyrosine nitrosylation of serum αSyn should have functional consequences. This protein contains 140 amino acids with four tyrosine residues (Tyr39 at the amine-terminus, and Tyr125, Tyr133, Tyr136 at the carboxyl-terminus), which are readily accessible for modification by nitrating agents[8]. Tyrosine residues of proteins can be differentially nitrosylated, leading to different effects, ranging from loss of activity until gain of function[16]. Regarding α-synuclein, it is known that nitrosylation at tyrosine residue 39 leads to reduced binding of αSyn to synthetic vesicles and to decreased rate of protein degradation[16]. Nitrosylation of tyrosine residues at both amine-terminus and carboxyl-terminus accelerates degeneration of dopaminergic neurons[19–21]. Nitrosylation at either Tyr39 or Tyr125/136 leads to enhanced aggregation of α-synuclein[18]. Thus, it is likely that enhanced nitrosylation of Tyr125/136 of serum αSyn could accelerate its aggregation in the nervous system. Of note is that there is substantial evidence that the conversion of αSyn from its soluble into the aggregated insoluble form is one of the key events of the pathogenesis of PD[24,25]. However, additional evidence exploring the causal relationship between alterations in function and the onset or propagation of a neurological disorder is required.

Conclusion

Our studies indicate the presence of halogenative and nitrosative stress in serum and, to a lesser extent, cerebrospinal fluid in early PD, characterized by excess of AOPP and 3-nitrotyrosine proteins, respectively. Metabolism of AOPPs and 3-nitrotyrosine proteins has been associated with several diseases, where blood phagocytes are overstimulated. Hence these types of stresses lead to the hypothesis that there is a maintained overstimulation of blood phagocytes in sporadic PD, which leads to excess formation of derivatives of hypochlorous acid and nitric oxide. Interestingly, nitrosative stress also affects serum N-αSyn, main component of Lewy bodies, hallmarks of PD. This protein shows an altered profile of tyrosine nitrosylation, a change that could represent an important pathogenic mechanism in PD, which requires further investigation.

Abbreviations list

AOPP, advanced oxidized protein product; CSF, cerebrospinal fluid; HAS, human seroalbumin; HOCL, hypochlorous acid; MPO, myeloperoxidase; N-αSyn, nitro-α-synuclein; PD, Parkinson's disease; ROS/RNS, reactive oxygen and nitrogen species

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